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Dissertação de Mestrado

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Dissertação orientada por Prof. Dr. Lounès Chikhi (IGC) e Prof Dr. Deodália Dias (FCUL)

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## Resumo

Vivemos num mundo altamente imprevisível no qual o Homem tem a capacidade de provocar alterações nas paisagens de forma cada vez mais intensa e com poucos limites. A diversidade genética atribui às espécies a capacidade de se moldarem às mudanças do seu ambiente, atribuindo-lhes um maior potencial de adaptação e sobrevivência (Reed e Frankham, 2003; Booy *et al*., 2000). É assim um factor de extrema importância na sobrevivência das espécies face a tais perturbações. Contudo hoje em dia esta tem vindo a ser comprometida, e uma das maiores causas é a perda e fragmentação dos seus habitats causada pela actividade humana (Schwitzer *et al*., 2013).

A ilha de Madagáscar é considerada um dos principais *hot-spots* de biodiversidade do planeta (Myers *et al.,* 2000). Contudo, ao mesmo tempo, é um local com um elevado impacto ambiental. A destruição causada pela actividade humana chegou a um ponto tão extremo nesta ilha que muitas das espécies endémicas se encontram à beira da extinção (IUCN, 2014). Uma preocupação mundial é a fauna endémica de lémures de Madagáscar, sendo considerados a maior prioridade de conservação de primatas do Mundo. Estes animais necessitam das florestas para sobreviver, no entanto, estima-se que apenas entre 10 a 20% da área da ilha permanece adequada à existência destes (percentagem total da área florestal) e nela a pressão humana ainda é uma realidade (Schwitzer *et al*., 2013; Myers *et al.,* 2000). Actividades como a agricultura, a produção de carvão e a extracção de produtos de uso local têm vindo a destruir habitats em grande escala. A actividade mineira que existe em Madagáscar tanto em pequena como em grande escala e o corte selectivo de árvores são também grandes causas de destruição (Schwitzer *et al*., 2013; Patel, 2007). Para acrescentar, a caça de lémures, apesar de ilegal, ainda acontece e compromete a sobrevivência de várias populações de lémures a nível local (Golden *et al.,* 2011; Golden, 2009).

Os lémures-rato (género *Microcebus*) são animais nocturnos, omnívoros que vivem nas florestas e são os primatas mais pequenos do mundo. O género *Microcebus* é dos mais abundantes em Madagáscar apresentando a distribuição mais ampla pela ilha (Rasoloarison *et al.,* 2013). Este género pertence à família *Cheirogaleidae* e contém pelo menos 21 espécies descritas até hoje (Rasoloarison et al., 2013; Radespiel *et al.,* 2012; Pastorini *et al.,* 2001). A espécie *Microcebus tavaratra* é uma espécie endémica de Madagáscar que pode ser encontrada em diferentes tipos de habitats, sendo a sua distribuição atribuída à ponta norte de Madagáscar (Salmona *et al.,* 2014). Esta

espécie é considerada Vulnerável de acordo com as designações da lista vermelha da IUCN (Andriaholinirina *et al.,* 2014). Um dos locais onde esta espécie pode ser encontrada é na região Loky-Manambato no norte de Madagáscar. Loky-Manambato é uma região protegida na totalidade desde 2012, sendo que alguns dos fragmentos florestais já eram protegidos desde 2005 (Schwitzer *et al.,* 2013, Salmona *et al.,* 2014). Contudo é uma região extremamente fragmentada com uma área florestal representada por apenas 17% da área total. Não se sabe quando esta fragmentação terá acontecido, contudo os fragmentos de floresta actualmente existentes sofrem todos de algum grau de destruição causada por actividades antropogénicas. Esta área é também atravessada por uma estrada nacional e pelo rio Manankolana. Várias espécies de lémures podem ser encontradas nesta região, incluindo a espécie *Microcebus tavaratra (*Salmona e Zaonarivelo, 2013).

No presente estudo a diversidade genética e a estrutura populacional da espécie *Microcebus tavaratra* da região Loky-Manambato foram avaliados. Mais especificamente, duas perguntas foram consideradas: (i) de que forma a diversidade genética se encontra distribuída por entre os fragmentos florestais e (ii) poderemos identificar os factores que geram diferenciação entre as populações nos diferentes fragmentos?

Para responder a estas perguntas DNA foi extraído de 113 biopsias de orelhas, colhidas em seis zonas de amostragem: Bekaraoka, Antsakay, Solaniampilana, Binara, Ambohitsitondroina e Benanofy. Não existem microssatélites criados especificamente para a espécie *Microcebus tavaratra*. Assim, vinte microssatélites criados para a espécie *Microcebus murinus* foram amplificados neste estudo. Os indivíduos de cada local de amostragem foram considerados como representantes de uma população. Para cada população as medidas de diversidade genética (He, Ho e Fis), diferenciação (Fst), estrutura (incluindo AMOVA) e o padrão de isolamento-por-distância foram calculados, utilizando os programas Genetix 2.05.2, STRUCTURE 2.3.4., ARLEQUIN 3.11 e GenAlex 6.5. Foi ainda avaliada a qualidade de amplificação de cada microssatélite de forma a identificar possíveis loci para os quais a amplificação tivesse sido alterada pela utilização de marcadores criados para outra espécie.

Cinco *loci* foram considerados como possivelmente problemáticos e não sendo possível averiguar isto de forma mais profunda na extensão deste estudo decidiu-se criar dois sets de análises, um com os 16 microssatélites (AS16) e outro no qual os 5 mencionados foram excluídos (AS11). Ambos os sets foram analisados em paralelo e os resultados foram apresentados neste

trabalho. Contudo apenas o set de análises AS11 foi considerado para as medidas de diversidade genética sendo que para o resto das análises apenas o set AS16 foi tido em conta.

Os resultados obtidos neste estudo mostraram que os indivíduos da espécie *Microcebus tavaratra* estudados ainda apresentam um elevado nível de diversidade genética, apesar de viverem numa região altamente fragmentada. As populações mostraram alguma diferenciação entre si, contudo não muito pronunciada. Isto poderá indicar a existência de corredores florestais a conectarem os fragmentos de floresta preservando a troca de genes entre as populações e/ou que o tamanho efectivo das populações é ainda grande o suficiente para que estas não sejam influenciadas pela deriva genética. As análises corridas com o programa STRUCTURE e a AMOVA sugeriram um agrupamento das populações em três grupos principais: Bekaraoka com Antsakay, Solaniampilana com Benanofy e Ambohitsitondroina com Binara. Um padrão fraco de isolamento-por-distância foi detectado, contudo não explicou este agrupamento nem a diferenciação entre as populações na totalidade. Esta por sua vez parece ser influenciada por uma combinação de factores. Por um lado notou-se a influência da falta de corredores florestais ligados aos afluentes do rio Manankolana ligando a maioria dos fragmentos a Ambohitsitondroina levando a uma diferenciação mais pronunciada desta população. Por outro lado a vila de Daraina e toda a actividade humana associada pareceu influenciar a diferenciação de Bekaraoka relativamente às outras populações e especialmente em relação a Binara, uma vez que este fragmento se encontra exactamente no lado oposto da vila. Estudos feitos na mesma área demonstraram que o rio Manankolana foi actua como um importante factor de diferenciação noutras espécies de lémures, como o *Propithecus tattersalli* (Queméré *et al,* 2010). Contudo aqui o rio Manankolana e a estrada nacional não pareceram actuar como barreiras ao fluxo genético entre as populações.

No espectro deste estudo os factores que influenciam a diferenciação das populações de *Microcebus tavaratra* não puderam ser examinados de forma mais detalhada. Não só o tempo foi limitado como se encontrou uma discrepância entre os tamanhos de amostra que dificultaram a interpretação dos resultados. Contudo os resultados obtidos neste estudo serão uma base para estudos mais amplos que determinem com mais precisão os efeitos da fragmentação nesta espécie. Tais estudos incluirão mais fragmentos de florestas e incorporar um maior número de marcadores e de indivíduos por cada fragmento. Deverão ainda ter o cuidado de incluir um número semelhante de indivíduos por população de forma a facilitar a interpretação dos resultados e obter melhores

conclusões. Isto será importante pois permitirá providenciar a informação necessária à criação de medidas de conservação mais robustas e que permitam uma melhor protecção desta espécie.

## Resumo (Abstract)

Madagáscar é um dos principais *hot spots* de biodiversidade do Mundo e ao mesmo tempo um local com um enorme impacto ambiental. A agricultura, corte de árvores e mineração de ouro destroem os habitats a uma velocidade tão elevada que muitas das espécies endémicas da ilha se encontram sob ameaça de extinção. Isto é uma realidade para muitas espécies de lémures da ilha. Neste estudo a diversidade genética e estrutura foram investigadas para seis populações do lémure-rato *Microcebus tavaratra.* Vinte microssatélites foram amplificados para 113 indivíduos mas quatro foram excluídos por serem monomórficos ou darem resultados duvidosos. A diversidade genética e diferenciação foram calculadas com base nas medidas He, Ho, Fis e Fst. A estrutura foi avaliada com uma inferência Byesiana (programa STRUCTURE 2.3.4) juntamente com o método de ΔK de Evanno *et al.,* 2005 e por uma AMOVA. O padrão de isolamento-por-distância também foi investigado. Os resultados obtidos neste estudo mostraram que apesar de viverem num ambiente fragmentado, as populações de *Microcebus tavaratra* ainda mostram um elevado nível de diversidade genética e um limitado nível de diferenciação. Isto sugere que o tamanho efectivo das populações ainda é elevado e/ou que o fluxo genético ainda se mantém entre os fragmentos florestais. Um fraco sinal de isolamento-por-distância foi detectado, contudo não pareceu ser o único factor a explicar a diferenciação. Parece haver uma influência da falta de corredores florestais a ligar Ambohitsitondroina aos outros fragmentos. Ao mesmo tempo, a vila Daraina juntamente com a actividade humana relacionada parecem estar a isolar a população de Bekaraoka dos outros fragmentos. O rio e a estrada nacional que atravessam a região não pareceram ter um papel importante na diferenciação entre as populações. Contudo, no espectro deste estudo não foi possível investigar de forma mais extensa os factores que influenciam a diferenciação entre as populações.

**Palavras-chave:** Madagáscar; Fragmentação; Diversidade genética; *Microcebus tavaratra*; Diferenciação.

## Abstract

Madagascar is one of the major hot spots of biodiversity in the world and at the same time a highly ecologically impacted place. The agriculture, tree cutting and gold mining destroy habitats in such a high rate that many of the endemic species of the island are now threatened with extinction. This is true for many lemur species of the island. Here genetic diversity and structure were assessed for six populations of the mouse lemur *Microcebus tavaratra*. Twenty microsatellite loci were amplified for 113 individuals, but four were excluded for being either monomorphic or provided unreliable results. Genetic diversity and differentiation were calculated based on He, Ho, Fis and Fst. Structure was inferred using a Bayesian approach (program STRUCTURE 2.3.4) together with Evanno et al, 2005 ΔK method and by an AMOVA. Isolation-by-distance was also assessed. The results obtained in this study showed that despite living in a highly fragmented environment, *Microcebus tavaratra* populations still present a high level of genetic diversity with limited level of differentiation between them. This suggested either that effective population sizes are very large and/or that gene flow is still maintained between forest patches. A weak isolation-by-distance pattern was detected, but it didn't seem to be the only factor influencing differentiation. There seems to be an influence from the lack of riparian forest corridors connecting Ambohitsitondroina to the other forests. At the same time the Daraina village and its related human activity appear to be isolating Bekaraoka from the other patches. The river didn't seem to play an important role in the differentiation between populations neither did the National road that crosses the region. However, in the extent of this study it wasn't possible to investigate to a greater extent the factors underlying the differentiation between these populations.

**Key words:** Madagascar; Fragmentation; Genetic diversity; *Microcebus tavaratra*; Differentiation.

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# 1. Introduction

We live in an unpredictable world where the human species is able to make incredibly quick and sudden changes in the natural landscapes, compromising the existence of many species (IUCN, 2014). In such a world, genetic diversity is of extreme importance to species survival. Once the loss of genetic diversity leads to heterozygosity decrease, populations will have a lower evolutionary potential (which means a lower potential to adapt to changing environments), lower fitness and thus face higher probability of extinction (Reed and Frankham, 2003; Booy *et al*., 2000).

Although natural landscape changes can influence genetic diversity, one of the major threats, not only to genetic diversity but to biodiversity as whole, is caused by anthropogenic activities. A well-known and worrying issue is the landscape modifications such as deforestation done either for survival reasons or for economic expansion, which leads to habitat loss and fragmentation (Schwitzer *et al*., 2013). Several studies have shown how habitat fragmentation influences genetic diversity not only in animal groups such as Fish, amphibians, birds, insects and mammals but also in plants (Balkenhol *et al.,* 2013-for mammals; Harrison *et al.,* 2012-for birds; Dixo *et al*., 2009-for amphibians; Aguilar *et al*., 2008-for plants; Yamamoto *et al*., 2004-for Fish; Joyce and Pullin, 2003-for insects). It is therefore of extreme importance to find ways to control human activities that may cause the destruction of habitats. This is possible with the implementation of conservation strategies. However, although feasible, these strategies are not possible without financial cost and being it a said reality, financial constraints pose a real difficulty in implementing conservation plans. Hence, as Myers and colleagues have suggested, it is important to find priority sites for which, as they wrote “we can protect the most species per dollar invested” (Myers *et al*, 2000).

Madagascar, Africa’s largest island (and the fourth largest in the world) is considered one of the major hot-spots for biodiversity in the world. Its complex topography and geographical location (south-east of the continent) generated a wide range of climates and environments, and being isolated from the continent, it created a huge diversity of mostly endemic fauna and flora in almost all taxonomic groups (Yoder and Nowak, 2006; Goodman and Benstead, 2003). According to Myers and colleagues this Island comprises 3.2% and 2.8% of all world’s plant and vertebrate species, respectively, with endemism ranging from 55-100% at the species level. It also surpasses that of any

other hotspot in the world at the genus and family level (Myers *et al.,* 2000). All of the above confer to Madagascar such an important value that makes it one of the world's highest priority areas for biodiversity conservation (Myers *et al*., 2000);

Despite its valuable endemic biodiversity and priority for conservation, Madagascar is also one of the most ecologically impacted countries worldwide, a reason why many species have already become extinct (Burney, 1999). It is estimated that between 80 and 90% of Madagascar's primary vegetation cover has already disappeared and many of the still present forest habitats are now fragmented (Myers *et al.,* 2000). As a consequence many of Madagascar's endemic species are on the verge of extinction (IUCN, 2014). Standing out as a global concern is Madagascar's primate fauna. More specifically the endemic lemur species, considered as the highest primate conservation priority in the world (Schwitzer *et al*., 2013). Given that only about 10 to 20 % of Madagascar's land area remains suitable for primates survival it is of extreme importance to understand in detail the effects of habitat degradation on these primates in order to create conservation strategies to protect them.

The present study is part of a major project which aims at assessing the effects of habitat loss and fragmentation across different lemur species thriving in the same region (e.g. Salmona *et al.,* 2014; Salmona et al., 2014b; Queméré *et al*, 2009, 2010, 2012; Viana *et al*., 2010; Pais, 2011). This study is an extension of the work of Isa Pais (Pais, 2011) where genetic diversity and structure for *M. tavaratra* species will be assessed based on microsatellite markers for six forest patches of the Loky-Manambato region of Madagascar.

### 1.1. Habitat loss and fragmentation in Madagascar

Evidence suggests that most of the changes that led to the habitat loss in Madagascar began with the arrival of humans about 4000 year ago (Dewar et al., 2013; Gommerv *et al.,* 2011). It is estimated that a great part of this loss, about 40%, happened more recently, between 1950s and 2000 (Harper *et al*., 2007). It is therefore considered that human activity has had a great part in the habitat destruction in Madagascar in the last centuries and specially now it is for sure a great threat to many lemur species throughout the island (Schwitzer *et al*., 2013).

One major anthropogenic deforestation driver is the slash-and-burn agriculture, which involves a process of cutting primary or secondary vegetation within a plot, waiting for it to dry and

then burning it to release the nutrients to the soil. Wood charcoal production for cooking and the extraction of forest products for local use which has been happening since the human arrival in the island, are also massive drivers of habitat loss (Schwitzer *et al*., 2013). Other activities include small and large-scale mining and selective logging which, although illegal, still exists and it's possibly the most spread-out activity throughout the country (Patel, 2007). To add, also illegal but driven by a survival need for food is Madagascar's wildlife harvest which is a major concern and it has been increasingly recognized to compromise the survival of lemur populations (Golden *et al.,* 2011; Golden, 2009).

Conservation strategies have been implemented throughout the country. By now, the island comprises more than 4.7 million ha of protected areas, which represents almost all of its remaining natural vegetation. However illegal activities still happen, like cutting trees of valuable timber species for example, still impacting the protected areas (Schwitzer *et al*., 2013). One important step towards controlling these illegal activities is the implementation of research projects in those areas. This will not only enable a better understanding of the effects of such disturbances in the lemur populations but also bring the presence of researchers to the protected areas and which represent a better surveillance and control of illegal activities (Salmona and Zaonarivelo, 2013).

### 1.2. Impacts of habitat loss in lemur species

Lemurs are forest dwelling animals and thus depend on the stability of the forests to thrive. Sadly, instead of living in healthy stable habitats, lemur populations are being hunted, suffering from indirect pressure of the sudden appearance of thousands of migrant miners in small areas where previously there was almost no human pressure, suffering from the destruction of their habitats and being forced to live in smaller, increasingly fragmented forest areas (Schwitzer *et al.,* 2013). As a consequence, since the arrival of men many lemur species have become extinct (although the exact causes are not entirely known), including for example the entire radiation of giant lemurs, which included at least 17 species described so far (Godfrey, 2002). Furthermore, despite all conservation efforts, illegal activities such as commercial hunting still happen occasionally, leading to the extinction of local populations (Schwitzer *et al*., 2013). So it is not surprising that by 2012, during a workshop involving more than 60 lemur experts, out of the 99 lemur taxa for which data was available, 24 were considered Critically Endangered, 49 Endangered, 20 Vulnerable, 3 Near Threatened and 3 Least

Concern. These values were obtained using the IUCN Red List criteria (IUCN, 2014; Schwitzer *et al*., 2013).

As it was already mentioned, habitat loss and fragmentation are common threats to species survival. Once a barrier impedes the connection between two populations, there will be consequences at the gene level (Reed and Frankham, 2003). This has been shown for many lemur populations throughout Madagascar's forests. Craul and colleagues collected ear biopsies for Milne-Edward's sportive lemur (*Lepilemur edwarsi*) populations in a very fragmented region on North-western Madagascar. They implemented two methods of analysis, one based on mitochondrial DNA (mtDNA) and the other on nuclear DNA (microsatellites). Their results showed an overall medium to low genetic diversity within populations. They also found evidence for a population collapse in the last hundred years, even in the largest forest analysed, having this species completely disappeared from the most isolated forest fragments (Craul *et al.,* 2009). Likewise, the effects of forest fragmentation have also been demonstrated for other species, like the red ruffed lemur (*Varecia rubra*) (Razakamaharavo *et al.,* 2009). On the other hand, a study was implemented for the golden-crowned sifaka (*Propithecus tattersalli*) from the Loky-Manambato region (North-eastern Madagascar), the same region of this study. The results showed that despite the high level of the forest fragmentation observed in the area, the populations still presented a high level of genetic diversity (Queméré *et al.,* 2009). However the authors noted that the high expected heterozygosity values in their samples were associated with a small number of alleles which has been suggested to happen in populations which were previously large and subjected to a demographic bottleneck (Nei *et al.,* 1975) and it has been linked to habitat fragmentation in other primates as well (e.g. Olivieri *et al.,* 2008).

The results of Olivieri and Queméré's studies show that the effects of forest fragmentation are not, to some extent, consistent, demonstrating that different species may respond differently to the same habitat disturbances. This may be due to their different social, foraging and breeding behaviours. However these comparisons must be made with caution once these studies used a different sampling design, different markers and where implemented in different regions, which may lead to biased comparison conclusions.

Some studies have shown the influence of habitat fragmentation on mouse lemur species. Guschanski and colleagues, for example, collected ear biopsies of the golden brown mouse lemur (*Microcebus ravelobensis*) from 9 sites of different sizes. Based on mtDNA markers they found

evidence of the effects of forest fragmentation in the differentiation and genetic diversity of the studied populations. According to their results populations within smaller forest fragments had reduced or completely eliminated genetic diversity as opposed to those within bigger fragments which showed high levels of diversity (Guschanski *et al.,* 2007). Negative effects of the decreasing size of forest habitats were also suggested for this species by Radespiel and colleagues, which also noted that not only natural but also anthropogenic barriers may compromise gene flow between social groups (Radespiel et al., 2008). Olivieri and colleagues also collected ear biopsies of *Microcebus ravelobensis* and two other mouse lemur species, the Bongolava mouse lemur (*M. bongolavensis*) and the Danfoss' mouse lemur (*M. danfossi*) throughout their entire distribution (North-western Madagascar). Based on microsatellite data they found evidence for the influence of forest fragmentation in population decline and genetic differentiation among populations. They suggested that this may have happened in the last 500 years (Olivieri *et al.,* 2008). On the other hand, Schad and colleagues showed only a limited effect of this disturbance on *Microcebus murinus* in the littoral forest fragments of South-eastern Madagascar (Schad *et al.,* 2004). Results are also not consensual for Microcebus species and more studies inferring the effects of habitat fragmentation on this genus are necessary.

It is important to increase the number of studies in all lemur species and apply a multi-species approach in order to enable comparison between them and a better understanding of the real effects of habitat destruction loss and fragmentation in lemur species as a whole. This study comes from that need and as mentioned above it will focus in the *Microcebus tavaratra* lemur species.

### 1.3. Mouse lemurs (*Microcebus* species)

Mouse lemurs (*Microcebus* sp.) are nocturnal, omnivorous forest dwelling animals and the world's smallest primates (<100g). They are one of the most abundant and widespread genera of lemurs, being found in all major forest habitats in Madagascar (Rasoloarison *et al.,* 2013; Olivieri *et al.,* 2007) (Fig.1). The genus *Microcebus* belongs to the family *Cheirogaleidae* together with the genera *Allocebus*, *Cheirogaleus*, *Mirza* and *Phaner* and it contains 21 species described to date (Rasoloarison et al., 2013; Radespiel *et al.,* 2012; Pastorini *et al.,* 2001). Some *Microcebus* species have a broad distribution range, such as *M. murinus* and others a regionally or even locally restricted distribution range, such as *M. griseorufus, M. berthae, M. myoxinus, M. ravelobensis, M.*

*sambiranensis* and *M. tavaratra* (Radespiel, 2006). As reviewed by Radespiel in 2006, *Microcebus* individuals are considered solitary foragers presenting, however, a regular contact with conspecifics within or outside the mating context. Their home range is generally smaller than five ha and their sleeping behaviour may be represented by the formation of stable sleeping groups of related females and solitary males (e.g. *M. murinus*), groups consisting of females and males (e.g. *M. ravelobensis*), or solitary sleeping (e.g. *M. berthae*). However, much of what is known about *Microcebus* species is based on the biology of *Microcebus murinus,* the most widespread species, and the ecology, distribution and population sizes of the remaining species are to a large extent unknown. Hence, further research is needed in order to better understand the foraging and social patterns of each species (Meyler *et al.,* 2012; Radespiel, 2006).

*Microcebus* species are sympatric with other nocturnal species and in most regions of Madagascar they are found coexisting with other *Microcebus* species, however without evidence for gene flow between them (Weisrock *et al.,* 2010). Being the most widely distributed genus in Madagascar, three main biogeographic models were designed to explain their distribution throughout Madagascar. There is no consensus between them yet, but nevertheless all three include the barrier effects of larger rivers, suggesting that these are an important factor in genetic differentiation (Olivieri *et al.,* 2007; Craul *et al.,* 2007; Wilmé *et al.,* 2006; Martin, 1995). In addition, Weisrock and colleagues presented an extensively and detailed species delimitation study of these genera, in 2010, and suggested that although recognized as different species, some of these lineages may not yet be reproductively isolated, highlighting the fact that lineage divergence may have occurred not long ago (Weisrock *et al.,* 2010).

Due to their short (one year) generation time which allows for demographic changes to quickly leave genetic signatures, and to their population sizes which are still large enough to have preserved some genetic diversity (essential to reconstruct those demographic changes), mouse lemurs represent a good model to study the consequences of habitat fragmentation (Olivieri *et al.,* 2008).

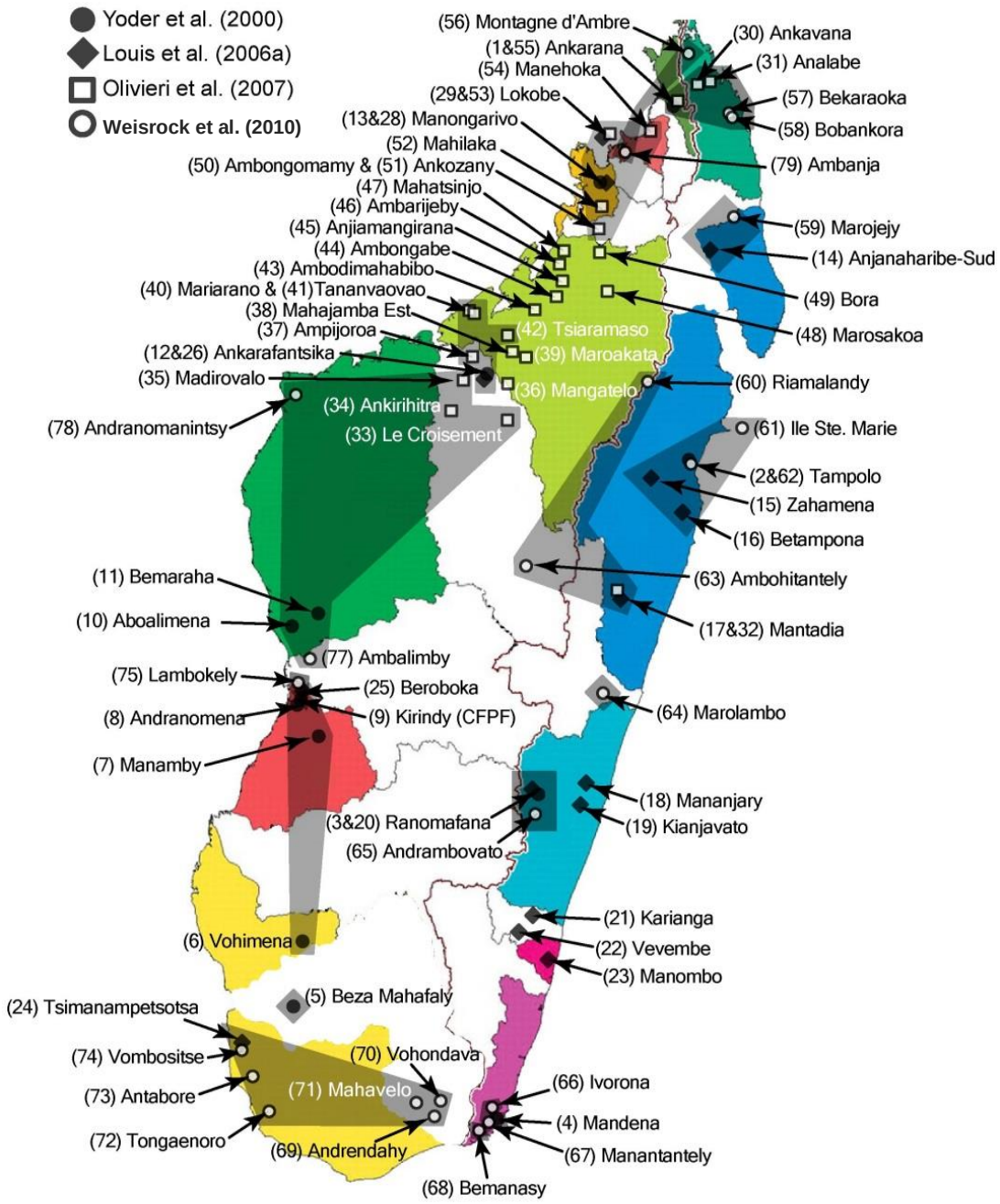


**Fig.1-** Distribution of *Microcebus* species known until 2010 and studied in Weisrock *et al.,* 2010 (Origin: Weisrock *et al.,* 2010).

### *1.3.1 Microcebus tavaratra*

*Microcebus tavaratra* is an endemic species of Madagascar. To date it has been found to inhabit the Loky-Manambato region (Daraina), Andavakoeira, Andrafiamena, Ankarana, Analamerana forest and up to the "Montagne des Français" in the north of Madagascar (Salmona *et al.,* 2014; Pais, 2011; Mittermeier *et al.,* 2010). *Microcebus tavaratra* can be found in different habitat types like tropical deciduous gallery forests or deep canyons sporting limestone pinnacles and cliffs (tsingy) in the Ankarana Special Reserve, lowland dry and transition humid forests of Daraina and possibly the tropical lowland montane forest in Montagne d'Ambre (Andriaholinirina *et al.,* 2014; Pais, 2011; Mittermeier *et al.,* 2010).

Their estimated population density ranges from 132 to 222 individuals /km$^2$ (Salmona *et al.,* 2014; Meyler *et al.,* 2012). This species inhabits severely fragmented habitats which are still decreasing in area and quality due to illegal activities such as logging, uncontrolled bushfires, cutting vegetation for charcoal and mining for sapphires (Andriaholinirina *et al.,* 2014).

All together provides this species with the conservation status of Vulnerable in the IUCN Red List. It is, however, a protected species and it is listed on Appendix I of CITES - Convention on International Trade in Endangered Species of Wild Fauna and Flora (Andriaholinirina *et al.,* 2014).

## 1.4. Microsatellites in the study of population genetics

Microsatellites, also known as simple sequence repeats (SSRs) or short tandem repeats (STRs) are motifs of one to six nucleotides repeated several times throughout the genome of Eukaryote beings (Kelkar et al., 2010). Having elevated mutation rates, they are typically polymorphic conferring variation between individuals, which is manifested as different repeat numbers (Guichoux et al., 2011). For their mutational quality, they play a major role in creating the genetic variation underlying adaptive evolution (reviewed by Bhargava and Fuentes, 2010). For this reason, microsatellites are widely used for the study of fingerprinting, parentage identification, genetic mapping and conservation and population genetics (Guichoux et al., 2011; Bhargava and Fuentes, 2010). Microsatellites are thus a powerful tool for inferring population patterns as well as demographic processes (Putman *et al.,* 2014).

### 1.5. The Loky-Manambato region (Daraina)

Loky-Manambato is a region in the north of Madagascar which, as the name indicates, is delimited by two main rivers, the Loky and the Manambato. It is a highly fragmented area with a total forest cover represented by only 17% of its total area (44,000 ha). However there is no knowledge as to when this fragmentation may have happened. What it is known is that in the present all forest patches suffer from some degree of human disturbance either for agricultural purposes and cattle grazing or hardwood harvesting and charcoal production (Salmona and Zaonarivelo, 2013; Queméré *et al.,* 2012).

The climate is seasonal, represented by a cool dry season from May to October and a hot, rainy season from November to April. This region has been fragmented into eleven forest patches which are formed mainly of lowland dry forest with primary and secondary dry deciduous and dry evergreen vegetation. However transition and humid forests can still be found (for example in Binara). The forest patches are surrounded by grazing pastures for zebu cattle, agricultural areas, human settlements, large grasslands or dry scrubs but also by a mixture of riparian gallery forests which, evidence suggests, may serve as a connection between patches (Salmona and Zaonarivelo, 2013; Queméré *et al.,* 2012). The Daraina region is also crossed by a large river, the Manankolana River, which flows from south to northeast, and by the RN5A unpaved national road which runs southeast to northwest (Fig.2).

In 2005 the Station Forestière à Usage Multiple – SFUM (Multiple Usage Forest Station) was implemented in the Loky-Manambato region and it only included some of the patches (as represented in fig.2) (Salmona et al., 2014). However since 2012 all the Loky-Manambato region became a protected area managed by the NGO Fanamby.

In this region several lemur species can be found. These include the Tattersall's sifaka (*Propithecus tattersalli*), the Sportive lemur (*Lepilemur milanoii*), the Sanford's brown lemur (*Eulemur sanfordi*), the Sanford's crowned lemurs (*Eulemur coronatus*), the Northern bamboo lemur (*Hapalemur occicentalis*), the Fat-tailed dwarf lemur (*Cheirogaleus medius*) and the Northern rufous mouse lemur (*Microcebus tavaratra*). The Montagne d'Ambre fork-marked lemur (*Phaner electromontis*) is also suspected to inhabit in this region as well as others that may remain to be discovered. Besides from the already mentioned anthropogenic pressures existing in this area, the

lemurs in this area are also increasingly threatened by unsustainable levels of hunting. It is thus not surprising that almost all the mentioned species are categorized as Endangered by the IUCN Red List, with the exception of *M. tavaratra* and *E. coronatus* which have the status Vulnerable and *C. medius* with is currently under the status of Least concern (Salmona and Zaonarivelo, 2013).

In this study samples were collected from six of the eleven forest patches of the region. These were Bekaraoka (62.48km$^2$), Benanofy (25.17 km$^2$), Solaniampilana (22.23 km$^2$), Ambohitsitondroina (38.32 km$^2$) and Binara (45.64 km$^2$). For analysis purpose, and for having too main separate sampling locations, Bekaraoka was considered as being two different populations: Bekaraoka south remained as Bekaraoka, and Bekaraoka north was named Antsakay. All forest patches are covered by the SFUM station (Salmona *et al.,* 2014) (Fig.2).

### 1.6. This study

The aim of this study is to assess genetic diversity and structure for *Microcebus tavaratra* species following the work carried out by Pais (2011) who confirmed that *M. tavaratra* was the species present in three forests, Bekaraoka, Binara and Solaniampilana of the Loky-Manambato region (Daraina). No evidence for sympatry with other *Microcebus* species was found although Pais (2011) recommended further investigation into this aspect. Pais study was also the first to assess genetic diversity for *Microcebus tavaratra* based on mtDNA and microsatellite markers. As an extension to Pais work, this study aims at increasing the number of microsatellite markers used, as well as the sample sizes and number of forest patches studied in the Loky-Manambato region. It is important to note that during the laboratory phase of this study mtDNA markers were also amplified for all individuals, however due to time constraint only analysis for the microsatellite data will be presented in this thesis.

Here, therefore two main questions are addressed: (i) how is genetic diversity distributed across forest fragments? and (ii) can we identify the factors generating differences between populations in different patches?

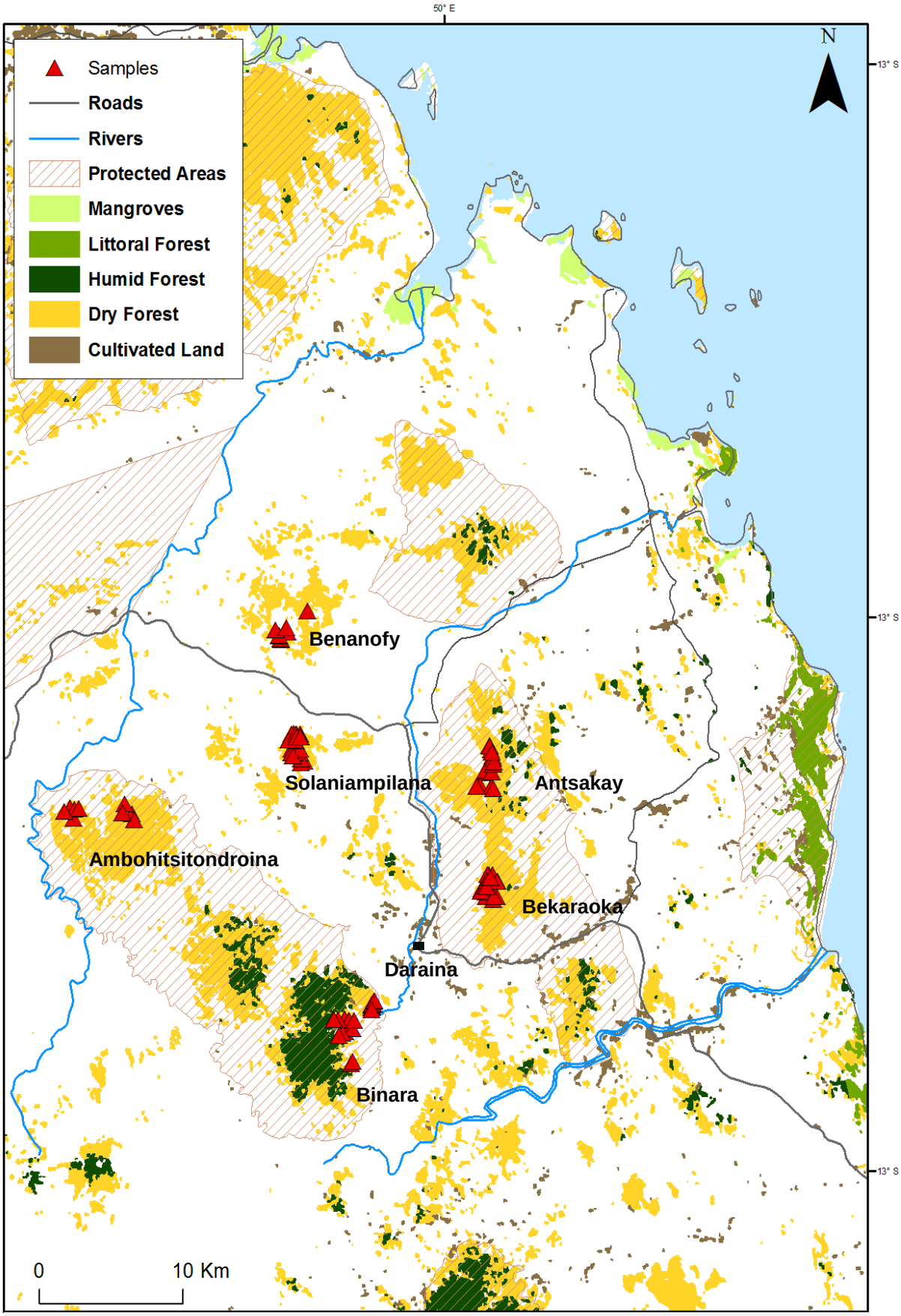


**Fig.2-** Map of the Loky-Manambato region (Daraina), with the six forest patches and sampling sites identified. This map was created using ArcGis. Note that the layer "protected areas" is not up to date and it represents the protected areas by the time of 2005.

# 2. Materials and Methods

## 2.1. Sample collection

Samples were collected in 2010 and 2011 by J. Salmona and various collaborator of an FCT-funded project led by L. Chikhi, from six sampling sites (Bekaraoka, Antsakay, Solaniampilana, Binara, Ambohitsitondroina and Benanofy) of the Loky-Manambato region (Daraina) in Northern Madagascar, using Sherman traps (H.B. Sherman Traps®) (Fig.2, Annex A). A total of 113 individuals (71 and 40 for 2010 and 2011, respectively) were genotyped and analysed in this study (Annex A). One to three ear biopsies were sampled from each *M. tavaratra* individual and stored in Queens Lysis Buffer (QLB, Seutin *et al.*, 1991) which allows for long term tissue and DNA preservation at room temperature.

## 2.2. DNA extraction

DNA extraction was performed for 113 ear biopsies. A blood and tissue Quiagen Kit was used for the extraction. Each biopsy was incubated in a solution of 300µl of digestion buffer (composed of 100mM EDTA, 100mM NaCl, 50mM Tris pH8 and 1%SDS), 20µl of Proteinase K at a concentration of 10mg/ml (Promega #V3021) and 20 µl 1M DTT (Dithiothreitol). A standard mammalian DNA isolation protocol was used as in previous studies by our group (Annex A; Annex C)

## 2.3. Microsatellite Amplification

In this study 20 polymorphic dinucleotide microsatellite markers were amplified (Table 1). Since there are no microsatellite loci specifically designed for *Microcebus tavaratra*, the microsatellites used here were originally designed for *Microcebus murinus* (Mm in the microsatellite labels*)*, and had already been successfully tested in other *Microcebus* species (with the exception of the locus Mm58 for which no data of such experiment was found) and, more recently, also for *M. tavaratra* (e.g. Pais, 2011- *M. tavaratra*; Olivieri *et al.,* 2008- *M. ravelobensis, M. bongolavensis and M. danfossi*; Hapke *et al*., 2003- *M.berthae*, *M.rufus* and *M. griseorufus*).

Each forward primer was marked with a fluorescent dye (Table 1). Considering the colour of the dye, the size and the annealing temperature of the primer six mixes were created for the PCR ampificaton. The optimization of these mixes was also part of this work. The mixes are identified in the table below where each colour on the “locus” row represents a different mix (Table 1). The

amplification occurred according to the following protocol: 10µl reaction containing 0.1µl of each primer (0.15 mM concentration), 5µl of My Taq HS Mix (Bioline-25045), 1µl of template DNA and water. The PCR cycle conditions were: initial denaturation of 4 minutes at 94 ºC, followed by 33 cycles of 30 seconds at 94ºC, 30 seconds at the primers annealing temperature (Table 1) and 30 seconds at 72ºC, and an extension of 7 minutes at 72ºC. Negative controls were used in every PCR reaction.

Genotyping was performed by the Genotyping Unit of the Instituto Gulbenkian de Ciências with ABI 3130 DNA equipment using LIZ labelled size standard ladder and the genotypes were checked and edited using GeneMapper® Software 5.

All the described methods were previously optimized by our group for their practicality and budget advantages and were chosen based on previous studies made by our group and associate groups, in order to facilitate posterior comparison (e.g. Pais, 2011).

**Table 1-** Primer information concerning all 20 loci used in the study

| Locus | Primer Sequence (5'-3') | AT | Dye | NA | GenBank Accession | Paper |
|---|---|---|---|---|---|---|
| Mm07 | AGTACCTAAGCCTGCCATTT | 50 | ROX | 5 | AF280082 | Radespiel *et al*., 2001 |
| Mm43b | CTA AAC TCC AAT ACA CAT ACC | 58 | FAM | 20 | AY154676 | Hapke *et al.,* 2003 |
| Mm60 | ACT GGA AAA TTT CAT TAC AAC AT | 54 | CY3 | 16 | AY154679 | Hapke *et al.,* 2003 |
| Mm03 | AGCCTCACTGTTTCAGTTGTGT | 55 | FAM | 15 | AF280081 | Radespiel *et al*., 2001 |
| Mm08 | CAGTTGGTGAATGGGCTAGG | 55 | CY3 | 29 | AF280083 | Radespiel *et al*., 2001 |
| Mm22 | GATATTTGCAGTGACGTCAAA | 58 | ROX | 16 | AY154670 | Hapke *et al.,* 2003 |
| Mm58 | GTTTGAACCCCCATAATATTCT | 54 | HEX | 2 | AY227663 | Hapke *et al.,* 2003 |
| Mm26b | TAA ATA ACC AAG TAA AGG GTT C | 58 | FAM | 10 | AY154671 | Hapke *et al.,* 2003 |
| Mm30 | GATGCTGAACCTCTGTCTG | 58 | CY3 | 15 | AY154672 | Hapke *et al.,* 2003 |
| Mm39 | TAC ACT CTG GGT TAC ATA AGA | 58 | ROX | 26 | AY154673 | Hapke *et al.,* 2003 |
| Mm51 | CTT GAG GAA GTC TCT GAG G | 58 | HEX | 12 | AY154677 | Hapke *et al.,* 2003 |
| Mm21 | TCAATGCATCAATTAACCACG | 58 | ROX | 16 | AY154669 | Hapke *et al.,* 2003 |
| Mm42 | CAT GGT TTC AGG TAC TCC C | 58 | FAM | 30 | AY154675 | Hapke *et al.,* 2003 |
| C1P3 | AGCCGAACACATTTCAGAGG | 50 | FAM | 21 | AF28007 | Radespiel *et al*., 2001 |
| Mm02 | TTAACAGGGCCTTCTCCTCAC | 53 | ROX | 10 | AF280080 | Radespiel *et al*., 2001 |
| Mm06 | CCT GCC TCA AAA TAA AAA AGA AAT | 48 | FAM | 16 | J420848 | Wimmer *et al*., 2002 |
| Mm40 | GAGAACAAGGATAGAATGTAAA | 58 | HEX | 13 | AY154674 | Hapke *et al.,* 2003 |
| Mm10 | GGGCTCCAATAGAGGCAATAA | 50 | HEX | 22 | AF280084 | Radespiel *et al*., 2001 |
| MmF3 | GCC CAA CGC TGA AGT AAG GAG | 48 | HEX | 18 | AJ420849 | Wimmer *et al*., 2002 |
| MmF6 | CAA CGG AGG GTA TTT TCA | 48 | ROX | 16 | AJ420850 | Wimmer et al. 2002 |

AT-annealing temperature; NA-number of alleles. Mixes are identified by colour in the "Locus" row.

### 2.4. Analysis

For the purpose of this study the 113 *M. tavaratra* individuals were considered as belonging to six populations according to their sampling forest patch (each forest patch was regarded as a different

population). Genetic diversity measures and departures from Hardy-Weinberg Equilibrium (HWE) were calculated for each population. Structure and Isolation-by-Distance (IBD) also assessed. For these analyses, four different programs were used: Genetix 4.05.2, a software designed to calculate several parameters commonly used in population genetics and carry out tests by permutation analyses (Belkhir *et al*., 2004); ARLEQUIN 3.11, which provides similar methods but also allows to carry out additional ones such as AMOVA (Laval and Schneider, 2005); GenAlex 6.5, an Excel add-in that provides a collection of Macros which allow a wide range of population genetic analyses and provides an easy way to carry out IBD analyses (Peakall and Smouse, 2012); and STRUCTURE 2.3.4, a program that implements a method that assesses population structure based on a clustering model that uses genotype data consisting of unlinked markers (Pritchard *et al*., 2000a).

### 2.4.1. Microsatellite choice

As mentioned before the microsatellite markers used in this study were not specifically designed for *M. tavaratra* species but for *M. murinus*. Although *M. tavaratra* and *M. murinus* belong to the same genus, it is important to identify and possibly exclude what in this study will be called “problematic loci”. At first 20 microsatellites were chosen from previous studies (Hapke et al., 2003; Wimmer et al., 2002; Radespiel et al., 2001). After amplification two were excluded for being monomorphic (Mm F6 and Mm60) and two others for showing amplification problems either for having too many missing data due to failure of PCR amplification (Mm10) or for resulting in suspicious readings which were not possible to decipher in this study (Mm06) (Table 1). Thus, the final data-set consisted of 16 loci. In order to check if there was any problematic loci (either for the presence of null alleles, allele dropout or false alleles) departures from Hardy-Weinberg Equilibrium (HWE) and $F_{IS}$ were calculated for each locus. HWE departures were calculated using the observed vs expected heterozygosity with a Q-square test for significance (using GenAlex 6.5) and significance of $F_{IS}$ was assessed with 10.000 permutations (using Genetix 4.05.2). Multiple comparisons were made for each population with and without the loci that seemed to be problematic in order to check for any major differences in the genetic diversity measures. Micro-Checker 2.2.3 software (Oosterhout *et al,* 2004) was also used to check for evidence of null allele and large allele dropout for each locus. In addition, comparisons were made between genetic diversity measures calculated for each locus on this study and other studies that used the same markers. This was important in order to check for any major difference between

studies, which could indicate possible errors of amplification. Pairwise linkage disequilibrium was estimated using Weir's correlation coefficient (Weir, 1979) and significance was assessed with 1000 permutations.

### 2.4.2. Genetic diversity

Genetic diversity was measured using the number of alleles per locus (NA), the mean number of alleles per population (MNA) the observed heterozygosity ($H_o$) and Nei's unbiased expected heterozygosity ($H_E$) per population (Nei, 1978). A linear regression was made to test for correlation between the mean number of alleles per population and the size of the population (number of individuals). $F_{IS}$ (intra-population fixation index) was calculated as a measure of departure from HWE and pairwise $F_{ST}$ as a measure of genetic differentiation between populations, both according to Weir and Cockerham (1984) and significance was accessed with 10.000 permutations. All the above mentioned analysis were made using Genetix 4.05.2, with the exception of the linear regression applied to the MNA which was done using Excel and the pairwise $F_{ST}$ which were calculated using ARLEQUIN 3.11.

### 2.4.3. Isolation by Distance

Isolation by Distance was assessed using GenAlex 6.5. Individual geographic distances were calculated by the program, based on GPS coordinates. Individual genetic distance was also calculated by the program. A Mantel test with 9999 permutations was performed using the two previously created matrices – individual geographical distance vs individual genetic distance.

### 2.4.4. Structure

As mentioned before the 113 individuals were grouped by forest (six populations). To test whether this was a valid grouping or if there was any different structure, a Bayesian clustering approach was implemented using STRUCTURE 2.3.4 software. Using a Markov Chain Monte Carlo (MCMC) approach the program groups individuals into clusters. Requiring a prior minimum and maximum number of expected genetic clusters (K), based on their genotypes and with no prior information on their sampling locations the program calculates the probability of each individual to be assigned to a particular cluster. Analysis were performed for K values between 1 and 9 (three more than the real

number as suggested by Evanno *et al.* (2005)), and for each K, 20 runs were performed with length of burnin period equal to 60.000 and 20.000 MCMC repeats after burnin. The admixture model was used with initial alpha value of 1.0 with this value being used for all populations, a maximum value for alpha 10.0 and Standard Deviation of 0.025. Allele frequencies were set to be correlated among populations and Fst values to be different for different subpopulations. A prior mean of Fst for populations was chosen to be 0.01, the prior SD of Fst for populations 0.05 and a constant lambda of value 1.0 (adapted from Queméré *et al.,* 2010; Olivieri *et al.,* 2008).

To determine the most probable K value, STRUCTURE HARVESTER (Earl and vonHolds, 2012) online server was used. This server implements the Evanno *ad hoc* summary statistic ΔK method (Evanno *et al*., 2005). It calculates ΔK based on the rate of change of the estimated likelihood between successive K values (L(K)) and using ΔK it suggests the most probable K applied to the input data.

Each individual was assigned to the group to which the posterior probability (q) was the highest. Individuals were only assigned to a cluster if this value was higher than 0.6 (as in Queméré *et al.,* 2010). Individuals for which the posterior probability was lower than 0.6, weren't attributed to any cluster. All the analyses were repeated for each cluster until no further substructure was found.

AMOVA

To further infer about the structure and differences within and amongst populations an AMOVA was performed using ARLEQUIN 3.11 software. Significance of results was tested under 10000 permutations.

NOTE: As in the Lab section, in order to facilitate comparison of results, all the analyses performed were done similarly to Pais, 2011, Queméré *et al*., 2010 and Olivieri *et al*., 2008.

# 3. Results

## 3.1. Microsatellite choice

In this study 16 polymorphic dinucleotide microsatellite loci were analysed. None of the loci showed systematic deviations from Hardy-Weinberg Equilibrium (HWE). However five loci (Mm02, Mm03, Mm21, Mm22 and Mm30) showed significant departure from HWE in more than two locus-population combinations, four of which (all but Mm03) showed evidence for null alleles. These five loci were considered as possible problematic loci. Two other loci (Mm26b and Mm40) showed HWE departure in one population each but no locus or populations seemed to stand out, so they were kept for all analyses. There was no evidence for linkage disequilibrium between any pair of loci, after Bonferroni correction; hence all 16 loci were kept and used for all the analyses. Furthermore, genetic diversity measures ($H_E$, $H_O$ and $F_{IS}$) were calculated for each of the 16 loci (Table 2) and compared to other studies that used the same markers. All values were in accordance with those from other studies with no major difference that could suggest any type of error (see comparisons in Annex B).

Running analyses with problematic loci can result in biased outputs. In order to prevent this from happening two sets of analyses were created, one with 11 loci (AS11), where the five possibly problematic loci were excluded and another set with all 16 loci (AS16). Both sets were analysed in parallel and in the end compared in order to check for any major difference, suggesting possible biased results. For some analyses, only AS11 was considered, whereas in others AS16 was used as well, as will be presented below.

Note that the names of the sampling sites were abbreviated with the following correspondence: BEK – Bekaraoka; SOL – Solaniampilana; BIN – Binara; ANTSK – Antsakay; AMBO – Ambohitsitondroina and BEN – Benanofy (see Annex A).

**Table 2 -** Genetic diversity per locus for each sampling site.

| Site | | BEK | | | | SOL | | | | BIN | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Locus** | **NA** | **Nº Indiv** | **$H_E$** | **$H_O$** | $F_{IS}$ | **Nº Indiv** | **$H_E$** | **$H_O$** | $F_{IS}$ | **Nº Indiv** | **$H_E$** | **$H_O$** | $F_{IS}$ |
| **C1P3** | 24 | 23 | 0.935 | 0.913 | 0.024 | 28 | 0.905 | 0.929 | -0.026 | 24 | 0.810 | 0.792 | 0.023 |
| **Mm02** | 9 | 23 | 0.819 | 0.696 | 0.154 | 29 | 0.687 | 0.552 | 0.200 | 23 | 0.715 | 0.565 | 0.213 |
| **Mm03** | 8 | 23 | 0.800 | 0.696 | 0.133 | 29 | 0.823 | 0.862 | -0.049 | 24 | 0.747 | 0.833 | -0.119 |
| **Mm07** | 6 | 23 | 0.812 | 0.783 | 0.038 | 28 | 0.803 | 0.821 | -0.023 | 24 | 0.750 | 0.667 | 0.113 |
| **Mm08** | 17 | 23 | 0.896 | 1.000 | -0.119 | 29 | 0.884 | 0.931 | -0.054 | 24 | 0.901 | 0.875 | 0.029 |
| **Mm21** | 13 | 20 | 0.844 | 0.750 | 0.114 | 17 | 0.738 | 0.706 | 0.045 | 17 | 0.809 | 0.412 | 0.499* |
| **Mm22** | 14 | 23 | 0.690 | 0.478 | 0.312* | 29 | 0.849 | 0.621 | 0.273* | 24 | 0.869 | 0.792 | 0.091 |
| **Mm26b** | 6 | 23 | 0.434 | 0.435 | -0.002 | 28 | 0.138 | 0.143 | -0.033 | 24 | 0.082 | 0.083 | -0.011 |
| **Mm30** | 6 | 23 | 0.564 | 0.565 | -0.002 | 28 | 0.642 | 0.429 | 0.337* | 24 | 0.629 | 0.375 | 0.409* |
| **Mm39** | 13 | 23 | 0.895 | 0.957 | -0.071 | 29 | 0.836 | 0.897 | -0.074 | 24 | 0.725 | 0.708 | 0.024 |
| **Mm40** | 12 | 22 | 0.687 | 0.6812 | 0.008 | 29 | 0.759 | 0.759 | 0.000 | 23 | 0.808 | 0.739 | 0.087 |
| **Mm42** | 19 | 20 | 0.873 | 1.000 | -0.150 | 29 | 0.837 | 0.759 | 0.095 | 23 | 0.707 | 0.783 | -0.109 |
| **Mm43b** | 13 | 23 | 0.821 | 0.913 | -0.115 | 29 | 0.578 | 0.517 | 0.107 | 24 | 0.747 | 0.792 | -0.061 |
| **Mm51** | 3 | 23 | 0.608 | 0.609 | -0.002 | 29 | 0.327 | 0.345 | -0.055 | 24 | 0.265 | 0.292 | -0.103 |
| **Mm58** | 13 | 23 | 0.810 | 0.870 | -0.076 | 29 | 0.883 | 0.862 | 0.024 | 24 | 0.869 | 0.917 | -0.056 |
| **MmF3** | 5 | 23 | 0.668 | 0.565 | 0.156 | 27 | 0.626 | 0.593 | 0.055 | 24 | 0.620 | 0.625 | -0.009 |
| **Site** | | **ANTSK** | | | | **AMBO** | | | | **BEN** | | | |
| **Locus** | **NA** | **Nº Indiv** | **$H_E$** | **$H_O$** | $F_{IS}$ | **Nº Indiv** | **$H_E$** | **$H_O$** | $F_{IS}$ | **Nº Indiv** | **$H_E$** | **$H_O$** | $F_{IS}$ |
| **C1P3** | 24 | 19 | 0.928 | 0.947 | -0.022 | 10 | 0.911 | 0.900 | 0.012 | 10 | 0.8833 | 0.7500 | 0.160 |
| **Mm02** | 9 | 19 | 0.717 | 0.579 | 0.197 | 10 | 0.553 | 0.200 | 0.650* | 8 | 0.767 | 0.500 | 0.364 |
| **Mm03** | 8 | 19 | 0.778 | 0.790 | -0.015 | 10 | 0.668 | 0.800 | -0.210 | 8 | 0.617 | 0.250 | 0.611 |
| **Mm07** | 6 | 19 | 0.791 | 0.790 | 0.002 | 10 | 0.726 | 0.700 | 0.038 | 10 | 0.7083 | 1.0000 | -0.455* |
| **Mm08** | 17 | 19 | 0.903 | 0.947 | -0.050 | 10 | 0.837 | 0.700 | 0.171 | 10 | 0.6500 | 0.5000 | 0.243 |
| **Mm21** | 13 | 17 | 0.838 | 0.706 | 0.162 | 10 | 0.884 | 0.900 | -0.019 | 8 | 0.850 | 0.625 | 0.278 |
| **Mm22** | 14 | 19 | 0.863 | 0.474 | 0.458* | 10 | 0.821 | 0.800 | 0.027 | 8 | 0.817 | 0.625 | 0.247 |
| **Mm26b** | 6 | 19 | 0.331 | 0.158 | 0.530* | 10 | 0.337 | 0.400 | -0.200 | 10 | 0.4417 | 0.3750 | 0.160 |
| **Mm30** | 6 | 19 | 0.630 | 0.579 | 0.083 | 10 | 0.653 | 0.600 | 0.085 | 8 | 0.775 | 0.750 | 0.034* |
| **Mm39** | 13 | 19 | 0.866 | 0.947 | -0.096 | 10 | 0.811 | 0.900 | -0.117 | 10 | 0.8583 | 0.8750 | -0.021 |
| **Mm40** | 12 | 19 | 0.822 | 0.790 | 0.041 | 10 | 0.579 | 0.700 | -0.223 | 10 | 0.9000 | 0.8750 | 0.030 |
| **Mm42** | 19 | 19 | 0.925 | 0.947 | -0.025 | 10 | 0.874 | 1.000 | -0.154 | 10 | 0.9250 | 1.0000 | -0.087 |
| **Mm43b** | 13 | 19 | 0.788 | 0.632 | 0.203* | 10 | 0.832 | 0.800 | 0.040 | 10 | 0.6167 | 0.6250 | -0.014 |
| **Mm51** | 3 | 19 | 0.494 | 0.579 | -0.179 | 10 | 0.268 | 0.300 | -0.125 | 10 | 0.2333 | 0.2500 | -0.077 |
| **Mm58** | 13 | 19 | 0.817 | 0.684 | 0.166 | 10 | 0.790 | 0.900 | -0.149 | 8 | 0.8583 | 0.7500 | 0.134 |
| **MmF3** | 5 | 19 | 0.588 | 0.684 | -0.170 | 10 | 0.621 | 0.400 | 0.368 | 10 | 0.6500 | 0.7500 | -0.167 |

NA-Number of alleles per locus; Nº Indiv-number of individuals per locus used by Genetix 4.05.2 to calculate the genetic diversity parameters; **$H_E$** -unbiased expected heterozygosity (Nei, 1978); $H_O$ -observed heterozygosity; $F_{IS}$ -intra-population fixation index (Weir and Cockerham, 1984). Significance of $F_{IS}$ was assessed with 10.000 permutations.* Values for which $p<0.05$.

### 3.2. Genetic diversity

**AS11**

The values for expected heterozygosity ($H_E$) didn't show a great variation, ranging from 0.662 for BIN to 0.767 for BEK. Intra-population fixation ($F_{IS}$) values ranged between -0.035 for BEK and 0.018 for ANTSK, however a 10 000 permutation test showed that none of the $F_{IS}$ values were statistically significant (Table 3).

The mean number of alleles ranged from 5.5 to 8.2 (Table 3), however a positive linear correlation was found between the MNA and sample size ($R^2 = 0.7546$). When AMBO and BEN (the populations with fewer individuals, 10 and 8 respectively) were excluded, no linear correlation was found ($R^2 = 0.0315$), hence only the other four patches were considered for this measure, where MNA values ranged from 7.1 for BIN and 8.2 for SOL (Table 3).

**AS16**

The expected heterozygosity was similar in all patches ranging from 0.691 for BIN to 0.760 for BEK. $F_{IS}$ values were particularly high for this set, ranging from 0.015 for AMBO to 0.097 to BEN. Statistical significance was assessed with 10000 permutations. Values were significant for SOL, ANTSK and BEN (Table 3).

The mean number of alleles ranged from 5.6 to 7.6. As in the first set a linear regression was implemented and it showed that this values were linearly correlated to sample size ($R^2 = 0.7712$). Excluding AMBO and BEN, again, the linear correlation wasn't detected ($R^2 = 0.0006$). Hence only the other four forest patches were considered for comparison with MNA for these ranging between 6.9 for BIN and 7.6 for SOL and for ANTSK (Table 3).

**Table 3 -** *M. tavaratra*'s genetic diversity parameters for each site.

| Analysis set | | AS11 | | | | AS16 | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Site** | **Nº Indiv** | **$H_E$** | **$H_O$** | **MNA** | $F_{IS}$ | **$H_E$** | **$H_O$** | **MNA** | $F_{IS}$ |
| **BEK** | 23 | 0.767 | 0.793 | 7.7 | -0.035 | 0.760 | 0.744 | 7.5 | 0.021 |
| **SOL** | 29 | 0.689 | 0.687 | 8.2 | 0.003 | 0.707 | 0.670 | 7.6 | 0.053* |
| **BIN** | 24 | 0.662 | 0.661 | 7.1 | 0.002 | 0.691 | 0.641 | 6.9 | 0.075* |
| **ANTSK** | 19 | 0.750 | 0.737 | 8.0 | 0.018 | 0.755 | 0.702 | 7.6 | 0.072* |
| **AMBO** | 10 | 0.690 | 0.700 | 5.5 | -0.016 | 0.698 | 0.688 | 5.6 | 0.015 |
| **BEN** | 8 | 0.702 | 0.704 | 6.0 | -0.003 | 0.722 | 0.656 | 5.7 | 0.097* |

$H_E$-Nei's unbiased expected heterozygosity (Nei, 1978), Ho-observed heterozygosity, MNA-Mean nº of alleles per site and $F_{IS}$ -intra-population fixation index (Weir and Cockerham, 1984). Significance of $F_{IS}$ was assessed with 10.000 permutations. *p-value<0.05

Pairwise Fst comparisons

Genetic differentiation between forest patches was measured by pairwise Fst comparisons.

**AS11**

Values were significant between all sites and ranged from 0.013 to 0.085 with the average of 0.060. All pairwise Fst values are presented in the table below. Statistical significance was tested with 10 000 permutations and significance is shown in the diagonal axis of the table (Table 4).

**AS16**

Here again all pairwise Fst values were significant and ranged from 0.024 to 0.084 with the average of 0.062. All pairwise Fst values are shown in the table below (Table 4). Significance was tested with 10 000 permutations and all values were statistically significant.

**Table 4 -** Estimated pairwise Fst value for all sites.

| AS11 | | | | | | | AS16 | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Site** | **BEK** | **SOL** | **BIN** | **ANTSK** | **AMBO** | **BEN** | **Site** | **BEK** | **SOL** | **BIN** | **ANTSK** | **AMBO** | **BEN** |
| **BEK** | 0 | *** | *** | *** | *** | *** | **BEK** | 0 | *** | *** | *** | *** | *** |
| **SOL** | 0.061 | 0 | *** | *** | *** | *** | **SOL** | 0.061 | 0 | *** | *** | *** | *** |
| **BIN** | 0.079 | 0.060 | 0 | *** | *** | *** | **BIN** | 0.084 | 0.053 | 0 | *** | *** | *** |
| **ANTSK** | 0.013 | 0.036 | 0.063 | 0 | *** | *** | **ANTSK** | 0.024 | 0.034 | 0.064 | 0 | *** | *** |
| **AMBO** | 0.048 | 0.058 | 0.079 | 0.052 | 0 | *** | **AMBO** | 0.072 | 0.061 | 0.061 | 0.070 | 0 | *** |
| **BEN** | 0.070 | 0.037 | 0.080 | 0.050 | 0.085 | 0 | **BEN** | 0.084 | 0.040 | 0.070 | 0.062 | 0.081 | 0 |

Fst calculated according to Weir and Cockerham (1984). *** p-value<0.05

### 3.3. Isolation by Distance

Isolation by distance was calculated based on individual genetic distances and individual sampling geographic distances.

**AS11**

Although statistically significant, a very week signal for isolation by distance was detected (R=0.172, p=0.000) (Fig.3).

**AS16**

As in the first Analysis set, a very week statistically significant signal for isolation by distance was detected (R=0.197, p=0.000) (Fig.3).

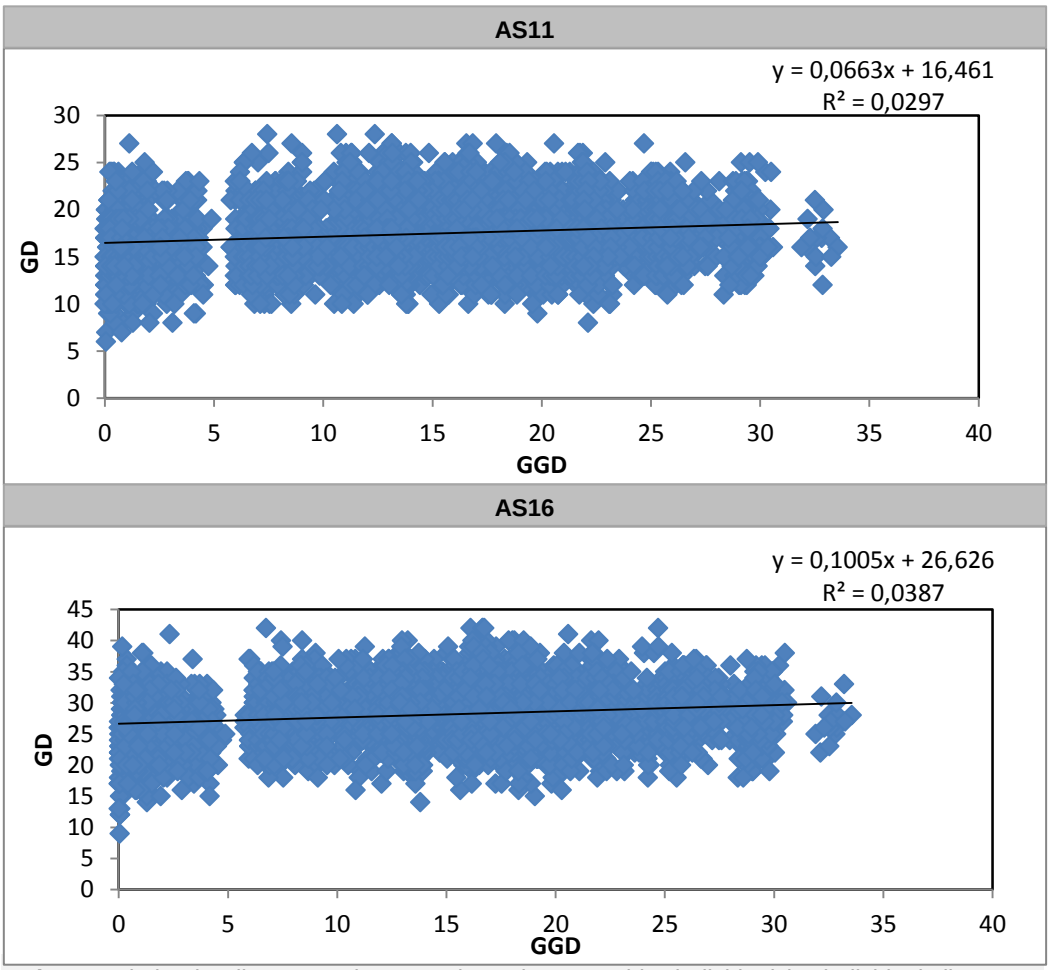


**Fig.3-** Isolation-by-distance using genetic and geographic, individual by individual distance matrices. AS11 - R= 0.1723 and p=0.000; AS16 - R=0.1968, p=0.000.

### 3.4. Structure

Applying the Bayesian approach in STRUCTURE 2.3.4 and Evanno ΔK method, the existence of 3 clusters was suggested for the two analysis sets since for both K=3 was associated to the highest ΔK value - (Evanno *et al.,* 2005) (Fig.5 for AS11 and Fig.6 for AS16).

**AS11**

Cluster 1 consisted of individuals from BEK and ANTSK, but also included two females from AMBO; cluster 2 was composed of individuals from SOL and BEN, plus one female from ANTSK and two females from AMBO; and cluster 3 consisted of BIN elements but also included one female from AMBO and one male from SOL. AMBO individuals were thus distributed across the three clusters, with higher memberships in cluster 2 and 3, however this population only has 10 individuals, a sample size too small for this higher membership to be considered significant. As in Queméré *et al.,* 2010, only individuals with a posterior probability (q) >0.06 were assigned to a cluster. This way, 9 individuals were not assigned to any of the three clusters (Tables 5 and 6).

**AS16**

Cluster 1 consisted mainly of individuals from BEK and ANTSK, with no individuals from other sampling sites assigned to it; cluster 2 consisted mainly of individuals from SOL and BEN with one female from AMBO; and cluster 3 consisted mainly of BIN and AMBO with no individuals from other sampling sites. Differing from the first set, here AMBO was fully attributed to cluster 3 whereas in AS11 it was distributed across the three clusters. A total of 6 individuals were left out for not showing a posterior probability higher than 0.6 (Tables 5 and 6).

**Table 5-** Proportion of membership of each population in the three clusters suggested by STRUCTURE 2.3.4.

| Analysis set | | AS11 | | | AS16 | | |
|---|---|---|---|---|---|---|---|
| | | Cluster | | | Cluster | | |
| Site | Nº Indiv | 1 | 2 | 3 | 1 | 2 | 3 |
| BEK | 23 | 0.901 | 0.056 | 0.044 | 0.936 | 0.035 | 0.029 |
| SOL | 29 | 0.070 | 0.854 | 0.076 | 0.034 | 0.910 | 0.055 |
| BIN | 24 | 0.041 | 0.047 | 0.912 | 0.018 | 0.027 | 0.956 |
| ANTSK | 19 | 0.819 | 0.124 | 0.058 | 0.858 | 0.084 | 0.059 |
| AMBO | 10 | 0.320 | 0.386 | 0.293 | 0.060 | 0.240 | 0.700 |
| BEN | 8 | 0.140 | 0.827 | 0.033 | 0.044 | 0.916 | 0.040 |
| Fst | - | 0.037 | 0.036 | 0.085 | 0.045 | 0.038 | 0.067 |

Fst-fixation index (mean value) for each cluster; NºIndiv-number of individuals belonging to each population.

**Table 6-** Total number and relative proportion of individuals of each site assigned to the 3 clusters.

| AS11 | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Total nº of Individuals | | | | Relative proportion of individuals | | | |
| Site | Nº Indiv | Clusters | | | Not assigned | Clusters | | | Not assigned |
| | | 1 | 2 | 3 | | 1 | 2 | 3 | |
| **BEK** | **23** | 23 | - | - | 0 | 100.0 | 0 | 0 | 0 |
| **SOL** | 29 | - | 28 | 1 | 0 | 0 | 96.6 | 3.4 | 0 |
| **BIN** | 24 | - | - | 23 | 1 | 0 | 0 | 95.8 | 4.2 |
| **ANTSK** | 19 | 16 | 1 | - | 2 | 84.2 | 5.3 | 0 | 10.5 |
| **AMBO** | 10 | 2 | 2 | 1 | 5 | 20.0 | 20.0 | 10.0 | 50.0 |
| **BEN** | 8 | - | 7 | - | 1 | 0 | 87.5 | 0 | 12.5 |
| **Total** | 113 | 41 | 38 | 25 | 9 | - | - | - | - |
| **AS16** | | | | | | | | | |
| | | Total nº of Individuals | | | | Relative proportion of individuals | | | |
| Site | Nº Indiv | Clusters | | | Not assigned | Clusters | | | Not assigned |
| | | 1 | 2 | 3 | | 1 | 2 | 3 | |
| **BEK** | **23** | 23 | - | - | 0 | 100 | 0 | 0 | 0 |
| **SOL** | 29 | - | 28 | - | 1 | 0 | 96.6 | 0 | 3.4 |
| **BIN** | 24 | - | - | 24 | 0 | 0 | 0 | 100 | 0 |
| **ANTSK** | 19 | 17 | - | - | 2 | 89.5 | 0 | 0 | 10,5 |
| **AMBO** | 10 | - | 1 | 7 | 2 | 0 | 10.0 | 70.0 | 20.0 |
| **BEN** | 8 | - | 7 | - | 1 | 0 | 87.5 | 0 | 12.5 |
| **Total** | 113 | 40 | 36 | 31 | 6 | - | - | - | - |

Nº Indiv-number of individuals sampled in each forest patch; Not assigned-individuals that were not assigned to any cluster with q>0.6 when K=3

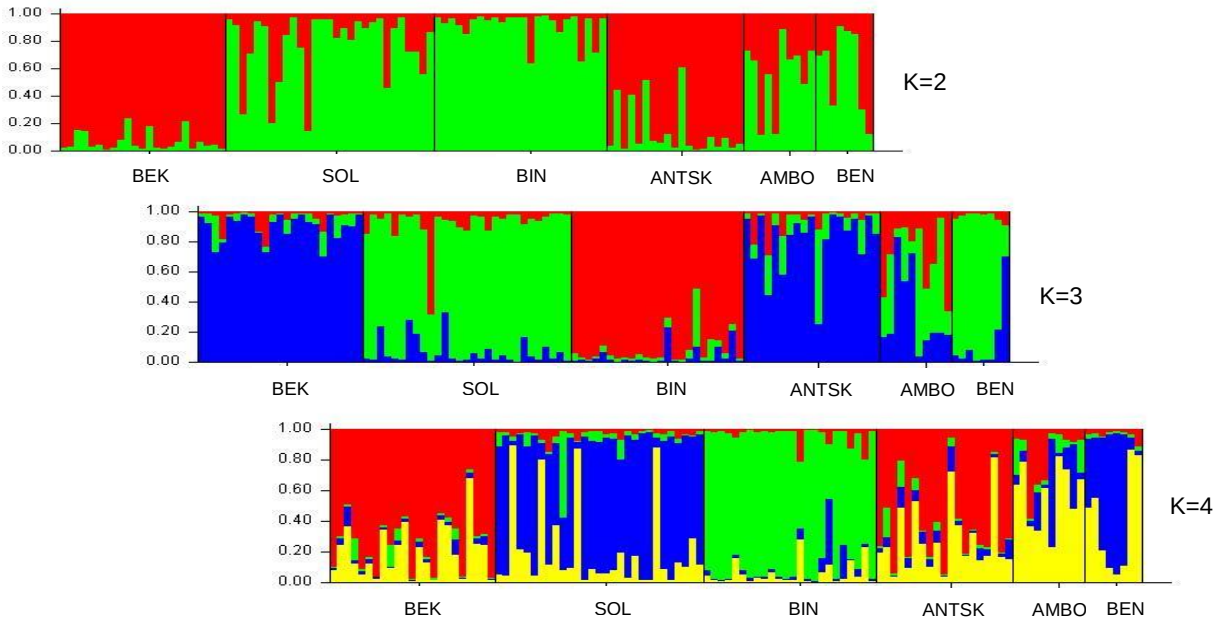


**Fig. 5- AS11** - STRUCTURE 2.3.4 Bar plot output for K=2; K=3 and K=4.
K=3 is the most probable number of clusters given that it is associated to the highest ΔK (Evanno *et al.,* 2005).

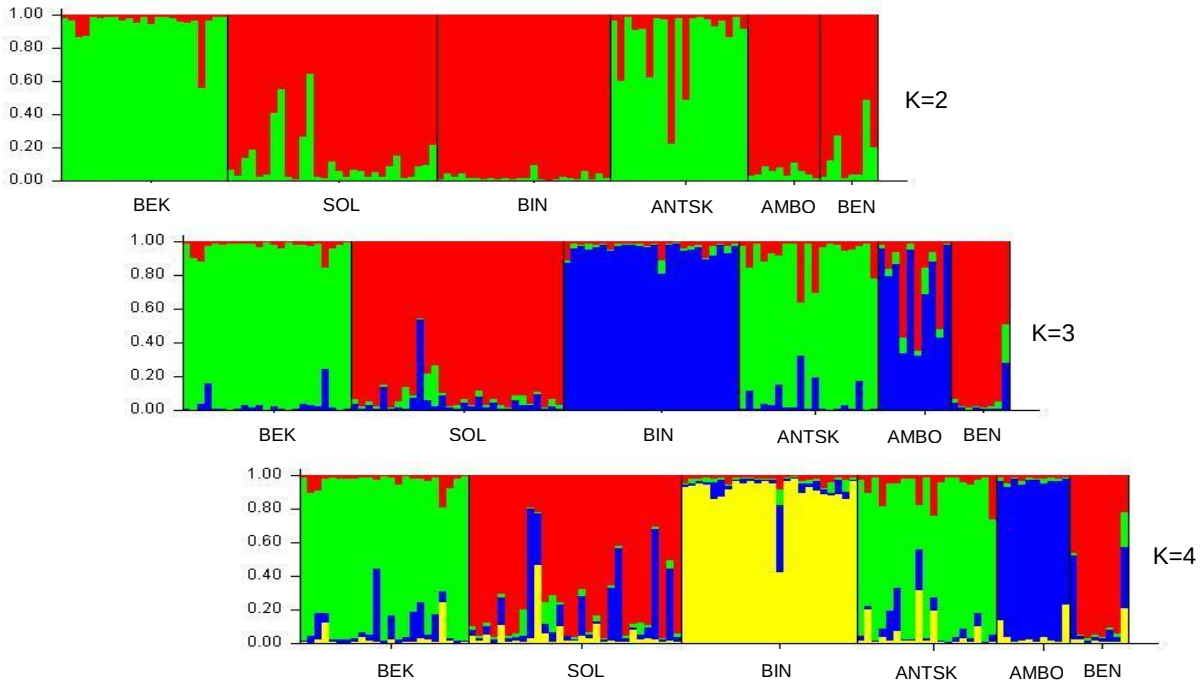


**Fig. 6- AS16 -** STRUCTURE 2.3.4 Bar plot output for K=2; K=3 and K=4.
K=3 is the most probable nº of clusters given that it is associated to the highest ΔK (Evanno *et al.,* 2005).

Evanno and colleagues have suggested that their method proposes the highest level of hierarchy so, after all individuals were assigned to one of the tree clusters the analysis was repeated for each cluster in order to check for further genetic structure. No substructure was found for any of the clusters in AS11 and AS16 (Evanno et al., 2005) (Fig.7 for AS11 and Fig.8 for AS16).

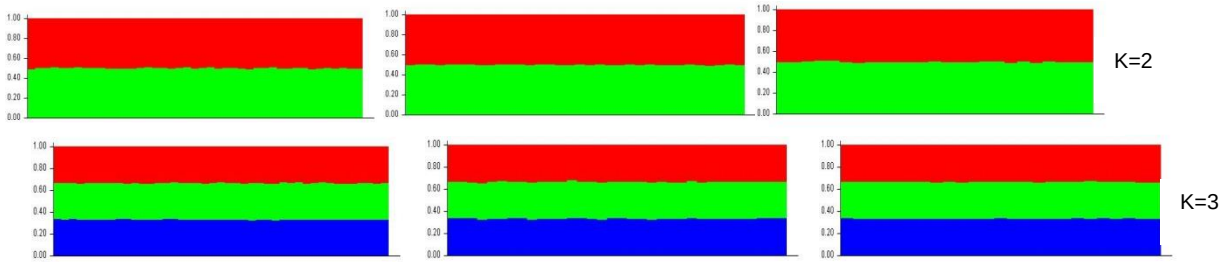


**Fig. 7- AS11 -** STRUCTURE 2.3.4 Bar plot output for cluster 1, 2 and 3 respectively when K=1 and K=2.

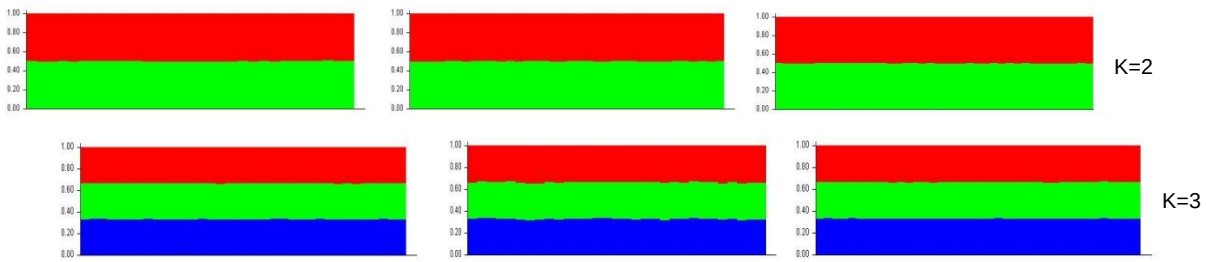


**Fig. 8- AS16** - STRUCTURE 2.3.4 Bar plot output for cluster 1, 2 and 3 respectively when K=1 and K=2.

AMOVA

The AMOVA test is another useful tool to assess population differentiation. It allows the user to gather the populations in different groups which is useful to infer for any differentiation pattern. Five different approaches were implemented when running the AMOVA, this is, populations were grouped in five different ways, as follows: Test 1 - one group with all populations; Test 2 – three groups with two populations each as suggested by STRUCTURE 2.3.4 (Group1- BEK and ANTSK, Group2- SOL and BEN; Group3- AMBO and BIN); Test 3 - two groups considering the division made by the National road (Group1- BEK, ANTSK and BEN; Group2- SOL, BIN and AMBO); Test 4 - two groups considering the Manankolana river's division (Group1- BEK and ANTSK; Group2- SOL, BIN, AMBO and BEN); Test 5 - two groups considering the type of habitat, i.e. dry forest vs humid forest (Group1- BIN-, Group2- SOL, AMBO, BEN, ANTSK and BEK) (Table 7).

**AS11**

The overall results from the AMOVA showed very little differentiation between populations given that for all tests the highest percentage of variation was attributed to "within populations". The values ranged from 93.31% for test 5 to 94.42% for test 1. Nevertheless, Fst, Fsc and Fct fixation values showed that some differentiation exists. Fst values for the comparisons "Among groups" and "Among populations within groups" ranged from 0.056 for test 1 to 0.067 for test 5. The Fsc values for comparisons "Among populations within groups" ranged from 0.039 for test 2, to 0.050 for test 3. All Fst and Fsc values were statistically significant with p-value<0.0001 (significance assessed with 10 000 permutations). Finally, the Fct values for comparisons "Among groups" were the highest for test 2 with 0.022, and the lowest for the test 3 with 0.0100. However none of the Fct values was statistically significant. The overall results show a greater differentiation between populations when these are grouped in the three clusters suggested by STRUCTURE, confirming this pattern. The second strongest result for differentiation between groups was for test 5 which considered forest type as a differentiation factor. The Manankolana river came in third place and the National road in last (Table 7).

**AS16**

The overall results from the AMOVA showed very little differentiation between populations with the highest percentage of variation attributed to "within populations". This percentage ranged from

93.30% for test 4 to 94.12% for test 1. Fst values for the comparisons “Among groups” and “Among populations within groups” ranged from 0.056 for test 1 to 0.067 for test 4. The Fsc values for comparisons “Among populations within groups” ranged from 0.039 for test 2 to 0.054 for test 5. All Fst and Fsc values were statistically significant with p-value=0.000. Finally, the Fct values for comparisons “Among groups” were the highest for test 2 with 0.025, and the lowest for the test 5 with 0.011. However none of the Fct values was statistically significant. As in the first Analysis set, the results show a greater differentiation between populations when these are grouped in the three clusters suggested by STRUCTURE. The second strongest result for differentiation between groups was for test 4 which considered the Manankolana river as a differentiation factor. The National road came in third place and the Forest type in last (although these last two showed very similar values for Fsc and Fct) (Table 7).

**Table 7-** Analysis of Molecular Variance-AMOVA for all sites.

| Analysis set | AS11 | | | | | | AS16 | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 - No grouping | | | | | | | | | | | | |
| **Source of Variation** | **d.f** | **Sum of sq** | **Var. comp.** | **% var** | **Fct/ Fsc** | **Fst** | **d.f** | **Sum of sq** | **Var. comp.** | **% var** | **Fct/ Fsc** | **Fst** |
| **Among populations** | 5 | 60.565 | 0.22691 Va | 5.58 | - | 0.0558 | 5 | 86.183 | 0.32752 Va | 5.82 | - | 0.0558 |
| **Within populations** | 220 | 845.126 | 3.84148 Vb | 94.42 | | | 220 | 1165.419 | 5.29736 Vb | 94.12 | | |
| 2 - STRUCTURE's output clusters | | | | | | | | | | | | |
| **Among groups** | 2 | 34.422 | 0.08856 Va | 2.17 | 0.0217 | | 2 | 50.011 | 0.13833 Va | 2.45 | 0.0245 | 0.0623 |
| **Among populations within groups** | 3 | 26.143 | 0.15398 Vb | 3.77 | 0.0385 | 0.0594 | 3 | 36.172 | 0.21362 Vb | 3.78 | 0.0388 | |
| **Within populations** | 220 | 845.126 | 3.84148 Vc | 94.06 | | | 220 | 1165.419 | 5.29736 Vc | 93.77 | | |
| 3 - National Road | | | | | | | | | | | | |
| **Among groups** | 1 | 17.009 | 0.04102 Va | 1.00 | 0.0100 | | 1 | 24.423 | 0.06060 Va | 1.07 | 0.0107 | 0.0621 |
| **Among populations within groups** | 4 | 43.555 | 0.20181 Vb | 4.94 | 0.0499 | 0.0595 | 4 | 61.760 | 0.29045 Vb | 5.14 | 0.0520 | |
| **Within populations** | 220 | 845.126 | 3.84148 Vc | 94.05 | | | 220 | 1165.419 | 5.29736 Vc | 93.78 | | |
| 4 - Manankolana River | | | | | | | | | | | | |
| **Among groups** | 1 | 19.142 | 0.06831 Va | 1.67 | 0.0167 | | 1 | 29.520 | 0.12514 Va | 2.20 | 0.0220 | 0.0670 |
| **Among populations within groups** | 4 | 41.422 | 0.18734 Vb | 4.57 | 0.0465 | 0.0624 | 4 | 56.663 | 0.25505 Vb | 4.49 | 0.0460 | |
| **Within populations** | 220 | 845.126 | 3.84148 Vc | 93.76 | | | 220 | 1165.419 | 5.29736 Vc | 93.30 | | |
| 5 - Forest type | | | | | | | | | | | | |
| **Among groups** | 1 | 19.126 | 0.08284 Va | 2.01 | 0.0201 | | 1 | 24.004 | 0.05985 Va | 1.06 | 0.0106 | |
| **Among populations within groups** | 4 | 41.439 | 0.19254 Vb | 4.68 | 0.0477 | 0.0669 | 4 | 62.179 | 0.30270 Vb | 5.35 | 0.0540 | 0.0640 |
| **Within populations** | 220 | 845.126 | 3.84148 Vc | 93.31 | | | 220 | 1165.419 | 5.29736 Vc | 93.59 | | |

Significance was accessed with 10000 permutations. p-value = 0.000 for variance components Vb, Vc, Fsc and Fst for all tests. Variance component Va and Fct values weren’t statistically significant for any of the tests. 11 loci were used for AS11 and 15 for AS16 with 0.05 allowed level of missing data.

# 4. Discussion

## 4.1. Microsatellite choice

The first step of the analysis performed in this study was to check for problematic loci. As the markers used in this study were designed for *Microcebus murinus,* amplification could be compromised, resulting in biased formation. Four loci were first identifies either because they could not amplify properly or because they were monomorphic and therefore could not be informative for population structure analysis. Five additional loci were then identified as potentially problematic and two sets of analysis were created, AS11 without those five loci and AS16 with all loci. Both sets were analysed in parallel in order to determine to what extent the excluded five loci could influence the results. The major difference in both sets was observed in the calculation of the genetic diversity measures (He, Ho and $F_{IS}$) and so only AS11 will be considered for those measures in this discussion. STRUCTURE 2.3.4 results were also different in both sets since with 11 loci Ambohitsitondroina was not attributed to any specific cluster and with 16 loci it was clustered with Binara. This second result makes sense since not even 50 years ago these two forest patches belonged to one major forest patch that now is fragmented into three (Queméré *et al.,* 2012). For the other analysis although differences were found no major difference provided evidence for biased results from the five possible problematic loci. In addition, the results obtained in this study for the single locus genetic diversity measures were in the same order as those obtained by other authors (Annex B). For this reason and because more loci provide more substantial data, this discussion will be based on the results provided by AS16.

## 4.2. Genetic diversity

Genetic diversity measures were assessed by using the polymorphic loci and the values shown here should therefore be overestimates. The mean number of alleles per site was used as well as the expected and observed heterozygosity. For living in a highly fragmented environment it was expected that signals of genetic diversity loss would be observed for the studied *Microcebus tavaratra* populations. However this was not the case. The average mean number of alleles per site was 7.4, and the average of the expected heterozygosity per site was 0.70. Observed heterozygosity values didn't differ much from the expected heterozygosity, with mean $F_{IS}$ of -0.001 (not significant) (AS11). These values are on the same order as those obtained by Queméré and colleagues for the Golden-crowned sifaka sampled in the same region (MNA= 6.3 and mean $H_E$ = 0.72) (Queméré *et al.,* 2010).

However, it is important to note that although Queméré and colleagues detected high levels of expected heterozygosity for the Golden-crowned sifaka, they also observed that those values were related with low mean number of alleles. This has been recognized as a signal of a past demographic bottleneck (Nei *et al.,* 1975) and actually, a few years later Queméré and colleagues did find this signal (Queméré *et al.,* 2012). The same was not observed here for the *Microcebus tavaratra* populations from the same area, which had all proximate and relatively high values of MNA. Microcebus species are known to be the smallest lemur species ever described whereas the Golden-crowned sifakas are medium-sized lemurs. One could think that differences in body size could lead to differences in the response of species to the same habitat disturbances, and that this could explain the difference in these results. However, Craul and colleagues detected signals for genetic diversity loss *for Lepilemur edwarsi*, a large-bodied lemur, similar to those observed by Olivieri and colleagues for three mouse lemur species subjected to deforestation and forest fragmentation on the same region (Craul *et al.,* 2009; Olivieri *et al.,* 2008). From their studies it is possible to see that the small and the large-bodied lemur species are both affected by habitat fragmentation in similar ways. If this applies across species, then the differences in size between *Microcebus tavaratra* and *Propithecus tattersalli* are not a probable explanation for these different results. *Propithecus tattersalli* are known to live in populations of approximately 800 to 5800 individuals with densities of 34 to 90 individuals/$km^{2,}$ (Queméré et al., 2010b; Vargas et al., 2002) whereas *Microcebus tavaratra* population sizes estimates vary from 44 000 and 74 000 with densities that range from 132 to 222 individuals/$km^2$ (Salmona et al., 2014). If one analyses the ratio between population size and population density it is possible to see that each Golden-crowned sifaka individual requires a larger area to survive (0.020 $km^2$/individual) than each *Microcebus tavaratra* (0.004 $Km^2$/individual). Such difference could explain different responses to habitat reduction and thus explain the different results. Other ecological aspects like nutritional preferences or preferred transiting substrate could also explain differences in responses to the same pressures. However due to the little that it is known about the ecology of *Microcebus tavaratra*, in the extent of this study these were not possible to further evaluate.

In this study it was also detected a relatively higher genetic diversity for the *Microcebus tavaratra* than for the three *Microcebus* species studied by Olivieri and colleagues (Olivieri *et al.,* 2008). In their study they obtained a mean number of alleles ranging from 4.38 to 6.50, from 3.63 to

5.00 and from 2.75 to 6.63 for *Microcebus ravelobensis, Microcebus bongolavensis* and *Microcebus danfossi*, respectively with an expected heterozygosity ranging between 0.52 and 0.71 for the three species. Here all monomorphic loci were excluded for the analysis of genetic diversity. This could explain the higher values observed here, however in Olivieri and colleagues study only one monomorphic locus was used for the species *M. ravelobensis* whereas for the other two species all loci were polymorphic, excluding this factor in the comparison (Olivieri *et al.,* 2008).

Comparisons between studies are not advisable when there are differences in the nature of the markers used, when the isolation method used for these markers is unknown or when the sampling design and analysis methods are not equal (Storfer *et al.,* 2007). However, the mentioned studies present similarities with this one. Queméré and colleagues study was implemented in the same area as this one, and Olivieri and colleagues worked with six of the same markers used here (and two different ones). In addition, the analyses implemented in this study were based on the ones implemented in these two studies allowing for some extent of comparison.

These findings suggest that despite living in an environment with a large fragmentation level, *Microcebus tavaratra* still appear to have a high level of genetic diversity.

### 4.3. Isolation by distance

A weak but significant signal for Isolation-by-distance was found in this study based on the individual genetic and geographic distances ($R=0.1968$, $p<0.0001$). When populations are isolated for a long time, it is expected that genetic drift erases any isolation-by-distance pattern (Olivieri *et al.,* 2008). Quemeré and colleagues also found an isolation-by-distance pattern for the Golden-crowned sifakas of the same region ($R = 0.449$, P value$<0.001$). The existence of this pattern for both species could be a hint that fragmentation happened not too long ago in this region. On the other hand, the weak isolation-by-distance pattern found here could be a sign that isolation for the *Microcebus tavaratra* populations happened so long ago that genetic drift has started to operate and erasing this pattern. However there is no clear conclusion about this so far as it was not possible to infer this to a deeper level.

## 4.4. Population differentiation and Structure

### 4.4.1. Pairwise Fst comparisons

The studied populations showed a relatively low but significant differentiation between them with an average pairwise Fst value of 0.062. The lowest Fst value was observed between Bekaraoka and Antsakay (both Fst= 0.024) and the highest value was observed for the pairs Bekaraoka-Binara and Bekaraoka-Benanofy (Fst= 0.084). The low differentiation between Antsakay and Bekaraoka makes sense since Bekaraoka and Antsakay are two sampling sites from the same continuous forest with no obvious barrier to gene flow such as savannahs or rivers between them. Differences between Bekaraoka and Benanofy may be easily explained by the pattern of isolation-by-distance, since those are two distant forest patches, together with the presence of the river Manankolana (Fig.2), as rivers have been recognized to serve as a major barrier to gene flow between populations (Queméré *et al.,* 2010, Craul *et al.,* 2007; Wilmé *et al.,* 2006). On the other hand, the major differentiation found between Bekaraoka and Binara cannot be explained by the Isolation-by-distance pattern. Bekaraoka and Binara are relatively close forest patches. Bekaraoka is actually closer to Binara than it is to Solaniampilana, Ambohitsitondroina and Benanofy (considering the geographical distances recorded with GPS coordinates taken at each sampling site). However two main barriers lie between these two forests. The Daraina village is situated between these two patches, with a great number of cultivated lands around it. The activity that comes from this village represents a strong human pressure that could prevent individuals of one population from contacting the other. Bekaraoka is in fact the forest patch that presented the highest Fst values for all comparisons (except with Antsakay) (Table 9). As it is visible on the map (Fig. 2), many cultivated lands surround Bekaraoka. Although *M. tavaratra* may cross these lands, these are usually linked to a higher human presence and pressure that could keep the *M. tavaratra* individuals away. Another factor is the Manankolana river that crosses between Bekaraoka and Binara, although this river also separates Bekaraoka from Benanofy it doesn't create the same level of differentiation. However the river in this region could be associated to more human activity coming from the nearby Daraina village and thus represent a stronger barrier to Binara. These two factors together may have led to a higher differentiation of the individuals inhabiting Bekaraoka which in contrast would have an easy contact with those from Antsakay. Solaniampilana was also strangely less differentiated from Antsakay, Benanofy and Binara than from Ambohitsitondroina. The isolation by-distance pattern doesn't explain this differentiation since the individuals sampled in

Ambohitsitondroina are much closer to those from Solaniampilana than from Binara and as close as Benanofy and Antsakay. However this could be explained by the existence of forest or bush corridors and riparian forests linked to the Manankolana river banks connecting Solaniampilana to Antsakay, Benanofy and Binara and not to Ambohitsitondroina (Fig.9). It is important to note that for this study an average of 24 individuals were sampled for Bekaraoka, Antsakay, Solaniampilana and Binara, but only 10 and 8 individuals were sampled for Ambohitsitondroina and Benanofy. A wider sampling size of both regions would be important in order to validate the viability of these results and infer with more clarity the reason for this differentiation.

**Table 4 (repetition of the right part)- AS16 -** Estimated pairwise Fst value for all sites

| Site | BEK | SOL | BIN | ANTSK | AMBO | BEN |
|---|---|---|---|---|---|---|
| **BEK** | 0 | *** | *** | *** | *** | *** |
| **SOL** | 0.061 | 0 | *** | *** | *** | *** |
| **BIN** | 0.084 | 0.053 | 0 | *** | *** | *** |
| **ANTSK** | 0.024 | 0.034 | 0.064 | 0 | *** | *** |
| **AMBO** | 0.072 | 0.061 | 0.061 | 0.070 | 0 | *** |
| **BEN** | 0.084 | 0.040 | 0.070 | 0.062 | 0.081 | 0 |

Fst calculated according to Weir and Cockerham (1984). ***p-value<0.05

Looking at these pairwise Fst values, differentiation between forest patches seems to vary according to different factors instead of following one clear pattern. In one hand, there is the lack of suitable riparian forest corridors connecting Ambohitsitondroina to the other patches, although with a more suitable connection to Binara through an intermediate forest patch (Antsahabe), which was not included in this study (Fig.9). On the other hand there is the existence of Daraina village and all its associated human pressure close to Bekaraoka, possibly isolating the individuals inhabiting this forest patch from the other ones with the exception of Antsakay (Fig.2). Isolation-by-distance also seems to be a contributing factor of differentiation between forest patches. However, although these may seem reasonable explanations, these factors alone may not explain all the differentiation patterns observed and in the present study those could not be more thoroughly examined.

In addition, although populations are differentiated from each other, differentiation has only reached a limited level, demonstrated by the limited Fst values. Queméré and colleagues have suggested that this could be a sign that fragmentation has happened not too long ago. On the other hand it could mean that fragmentation has happened so long ago that this species have adapted to

this environment and the low levels of differentiation are kept by the contact between migrants that can easily travel among patches (Queméré *et al.,* 2010).

### 4.4.2. STRUCTURE

There was no well-defined consensus between Fst differentiation values and the clusters suggested by Structure. Using the method suggested in Evanno *et al.,* 2005, three clusters were considered as the most probable structure for the data, with no further substructure. Bekaraoka was gathered with Antsakay, Solaniampilana with Benanofy and Binara with Ambohitsitondroina (Table 10). Here again there is no simple explanation for this separation. If purely isolation-by-distance was influencing the differentiation of these populations, it would be expected that Ambohitsitondroina was gathered with Solaniampilana since this is the closest patch. This differentiation suggests again some kind of barrier to gene flow between Solaniampilana and Ambohitsitondroina, and between Bekaraoka and Binara. These results reinforced the two previous presented hypotheses: the lack of suitable forest corridors connecting Ambohitsitondroina to the closer forest patches and the human pressure coming from Daraina village and associated activities between Binara and Bekaraoka as isolation factors shaping the differentiation of these populations. If so, then isolation-by-distance could also be playing its part since besides from Ambohitsitondroina, Solaniampilana is closer to Benanofy and Antsakay actually belongs to the same forest patch than Bekaraoka (Fig.2). In addition if one looks at images from 60 years ago (not presented here) it is possible to see that Binara and Ambohitsitondroina both belonged to one larger forest not too long ago (Queméré et al., 2012). Hence it is probable that the individuals from these two sampling sites, even if isolated from each other by know still share similarities from that time which could explain the STRUCTRE results. Also to be considered is the geography of the terrain. Some landscape features can be more appealing to this species and lead them to travel to more distant patches rather than to the closer ones. Biogeographical and past-history studies should be implemented to the *Microcebus tavaratra* of this region in order to understand how this species reacts to different landscape features and to infer possible past population dynamics that could explain their differentiation.

AMOVA

To further infer about differentiation patterns underlying the studied populations, several AMOVA tests were performed considering different possible barriers to gene flow. These were: the National road,

the River Manankolana, and forest type (Fig.2). Two additional AMOVA tests were performed, one considering the three clusters suggested by STRUCTURE 2.3.4 and another considering no barrier at all (all populations in the same group). In all cases the major percentage of variation was attributed to "within populations" with an average of 93.77% across the five tests, suggesting a low level of differentiation between the populations. Nevertheless differentiation exists and the test that better explained the structure underlying this differentiation was the one where populations were grouped according to the three clusters suggested by the STRUCTURE 2.3.4 analysis (Fct= 0.0245 and Fsc= 0.0388). The separation related to Manankolana river also appeared to explain the structure of the six populations although not as evidently as the three clusters mentioned above (Fct= 0.0220 and Fsc= 0.0460). The National road and the Forest type seemed to have a very similar influence in population differentiation and both came out as the weakest factors (Table 12).

Queméré and colleagues identified the river Manankolana as a major barrier to dispersal for the Golden Crowned Sifaka in the Daraina region, and a strong factor shaping genetic differentiation between populations. Rivers are recognized to serve as a barrier to gene flow, and have been demonstrated to play a major role in the differentiation of several lemur species, including mouse lemurs (Olivieri *et al.,* 2007; Craul *et al.,* 2007; Guschanski *et al.,* 2007; Louis *et al.,* 2006). However, it has also been demonstrated that not all rivers act as barriers to gene flow in this genus and that this is also dependent on the river's geographical features, such as the location of its origin (Olivieri *et al.,* 2007). Manankolana river separates Antsakay and Bekaraoka from the other forest patches. In fact, the STRUCTURE 2.3.4 analysis showed a potential influence of the river on the *Microcebus tavaratra* of this region since Antsakay and Bekaraoka, both on the same side of the river, where clustered together and the other two clusters included the forests on the other side. The AMOVA test considering the possible influence of the river was also the second strongest in terms of differentiation among groups. The Manankolana river is dry for most of the year, however as Queméré and colleagues suggested, it could still serve as a barrier to gene flow in two different ways: 1-if mating season corresponds to the rainy season when the river is full, then mating with individuals from the other side of the river should not be possible; 2-even when the river is dry, the presence of humans coming from nearby villages could inhibit the animals to cross it (Queméré *et al.,* 2010). For *Microcebus tavaratra*, however, the second hypothesis doesn't seem plausible as Microcebus is a

nocturnal species and their activity occurs mainly during the night and by that time human activity around the river must be scarce.

Another possible barrier to gene flow could be the National road, which separates Antsakay, Bekaraoka and Benanofy from Ambohitsitondroina, Binara and Solaniampilana. The data obtained in this study is not consistent with this hypothesis since one of the obtained clusters includes Benanofy and Solaniampilana which are separated by the road. One possible explanation for this is that, being nocturnal animals, Mouse lemurs probably move around the road during the night, which is a time when they face a lower probability of encountering a car crossing it or any human activity around it. This makes it more probable for them to cross the road and contact with individuals from the other side. On the other hand, Queméré and colleagues also observed that for the Golden-crowned sifaka, STRUCTURE analysis clustered Bekaraoka with Bobankora, a forest patch south of Bekaraoka and on the other side of the National road (Queméré *et al.,* 2010). This could mean that this National road is not a barrier to gene flow in lemur species of the Loky-Manambato region. More studies are needed in order to understand to what extent these barriers may influence genetic variation and differentiation in these populations and what are the effects of the forest fragmentation that these animals have been facing.

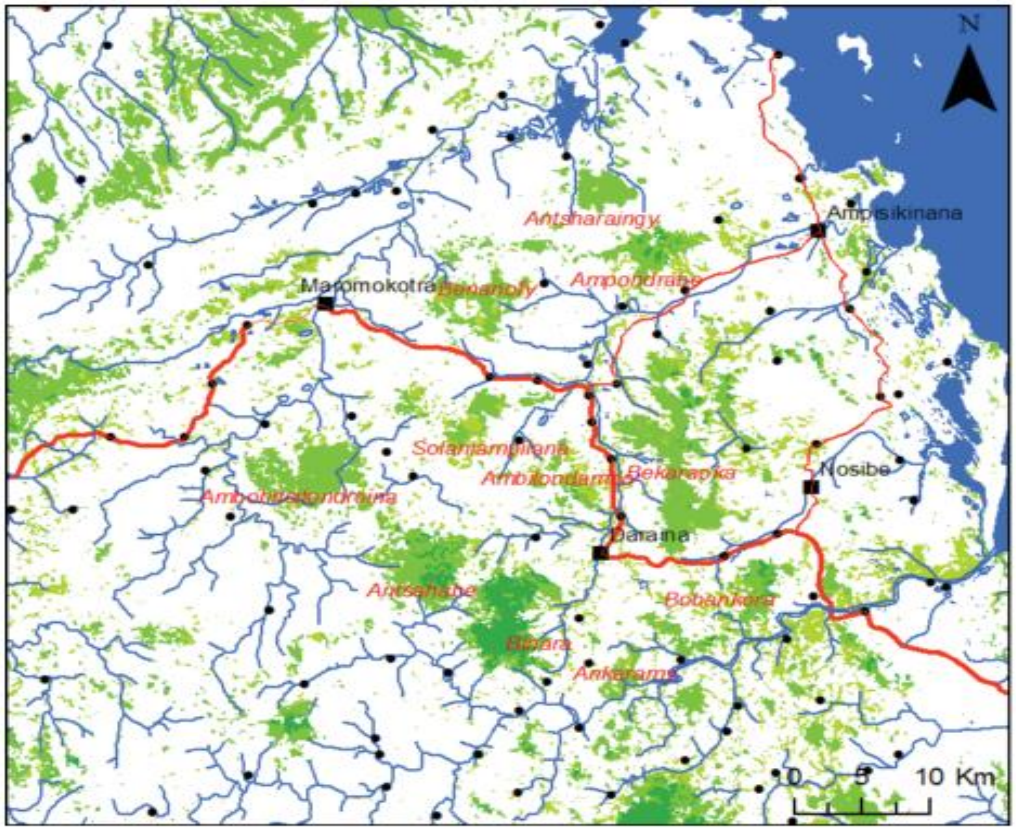


**Fig.9-** Map of the Loky-Manambato region (Salmona and Zaonarivelo, 2013)

### 4.5. This study as an extension to Pais work (2011)

This study was an extension to Isa Pais work (2011). Here three additional forest fragments corresponding to 60 new individuals have been analysed using more markers. Here genetic diversity and differentiation patterns were assessed based on Microsatellite markers, for *Microcebus tavaratra* of six forest patches of the fragmented Daraina region in the north of Madagascar. The nDNA diversity measures presented here are in the same order as those obtained by Isa Pais, although with some differences that where expected due to the different number of loci used (Table 13) (Pais, 2011). In the analysis performed with STRUCTURE 2.3.3, however, Pais suggested that Solaniampilana, Bekaraoka and Binara belonged to the same cluster. Due to the differences in the number of loci used in both studies it is difficult to compare both studies, and this difference most probably comes from this factor.

**Table 13-** Genetic diversity measures for Microcebus tavaratra obtained by Pais, 2011 and in this study

| | Pais, 2011 | | | | This study | | | |
|---|---|---|---|---|---|---|---|---|
| **Site** | **MNA** | **He** | **Ho** | **Fis** | **MNA** | **He** | **Ho** | **Fis** |
| **BEK** | 5.8 | 0.727 | 0.739 | 0.016 | 7.7 | 0.767 | 0.793 | -0.035 |
| **SOL** | 6.8 | 0.782 | 0.782 | 0.043 | 8.2 | 0.689 | 0.687 | 0.003 |
| **BIN** | 7.8 | 0.661 | 0.746 | 0.661 | 7.1 | 0.662 | 0.661 | 0.002 |

He-unbiased expected heterozygosity (Nei, 1978); Ho-observed heterozygosity; Fis-intra-population fixation value; MNA-mean number of alleles. None of the Fis values are statistically significant.

It is interesting to see that from the genetic diversity measured by Pais based on mtDNA these three populations showed a higher differentiation between them. Especially interesting is the fact that Solaniampilana and Binara seemed to be more differentiated from each other than from Bekaraoka (Table 14). This goes against what was found in this study. It is difficult to interpret these different results, since they are based on different markers. Especially because mtDNA is only transmitted maternally, reflecting female migration patterns and changes in genetic diversity measures may happen in different time lengths than those in the nuclear DNA. It is possible that these similarities in the mtDNA found by Isa Pais have been established a long time ago, before Bekaraoka became more pressured by human activity and more isolated from the other forest patches. If so, then microsatellite loci could have undergone quicker changes that could signal this isolation. However this is just a theory that could not be further investigated but hopefully will be cleared out in the span of this project.

**Table 14**- mtDNA based Pairwise Fst values for three populations obtained by Isa Pais (Pais, 2011).

| Site | BEK | SOL | BIN |
|---|---|---|---|
| **BEK** | 0 | | |
| **SOL** | 0.339 | 0 | |
| **BIN** | 0.549 | 0.763 | 0 |

Values obtained by the concatenation of pairwise Fst results for COII, *cyt b* and d-loop regions of the mtDNA

# 5. Conclusion

The overall results from this study showed that, despite inhabiting in a fragmented region all populations showed high and similar levels of genetic diversity. It was clear from the results obtained here that the populations from different forest patches are differentiated from each other, even if with low levels of differentiation. The low levels of differentiation suggest that forest patches are still connected and/ or that populations are still large enough to be little influenced by genetic drift.

The low levels of differentiation combined with the isolation-by-distance patterns may suggest that fragmentation has happened not too long ago. On the other hand it may mean that it happened long enough for genetic drift to begin to erase isolation-by-distance and even if populations have become isolate, low differentiation levels may be explained by the regular contact with migrants from other populations.

Differentiation between populations seems to be influenced by a combination of factors and not by a simple isolation-by-distance pattern. For instance, this pattern doesn't explain theslightly higher that expected differentiation that Ambohitsitondroina and Bekaraoka show with some of the nearby patches. What is hypothesized here is that there may be a lack of suitable forest corridors connecting Ambohitsitondroina to other forest patches, with the exception of Binara to which it should have a good connection via an intermediate forest patch (Antsahabe) (Fig.9). Bekaraoka may have become isolated due to the intensive human activity around it related to the Daraina village. The river or the National road didn't seem to play a strong role shaping genetic differentiation across the populations of *Microcebus tavaratra* (Fig.2). It is however, important to note that the Loky-Manambato region includes eleven forest patches and that the analyses made with only six patches may not be enough to extract any solid conclusions.

The factors influencing differentiation among the six analysed populations could not be assessed to their whole extent. Little is still known about this species and how, or even if, it has been influenced by the effects of fragmentation of habitats as a whole. To better understand the patterns of differentiation described in this study and further investigate about the effects of habitat destruction in this species a wider data set would be needed, with more markers and including more forest patches. Also, it is advisable to include a similar number of individuals of each forest in these studies so that

the results can be more consistent and clear. This was not possible in the present study. However, the data obtained here hopefully will serve as a good basis for such studies in order to enable the implementation of stronger conservation measures and preserve what has the right to be preserved.

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## Annex A

**Table 15-** Information about all Microcebus tavaratra individuals from this study.

| Site | POP | Forest | Forest_code | GPS_SN | GPS_EO | Sex | ID-ind_Lab | Year | ID Indv |
|---|---|---|---|---|---|---|---|---|---|
| Daraina | 1 | **Bekaraoka** | BEK | -13,1732405 | 49,7031348 | F | A01_2010 | 2010 | **A01** |
| Daraina | 1 | **Bekaraoka** | BEK | -13,1704576 | 49,7028922 | M | A02_2010 | 2010 | **A02** |
| Daraina | 1 | **Bekaraoka** | BEK | -13,1746721 | 49,7078386 | M | A03_2010 | 2010 | **A03** |
| Daraina | 1 | **Bekaraoka** | BEK | -13,15983 | 49,70462 | F | A04_2010 | 2010 | **A04** |
| Daraina | 1 | **Bekaraoka** | BEK | -13,1598 | 49,70724 | M | A06_2010 | 2010 | **A06** |
| Daraina | 1 | **Bekaraoka** | BEK | -13,1635951 | 49,7102932 | M | A08_2010 | 2010 | **A08** |
| Daraina | 1 | **Bekaraoka** | BEK | -13,168586 | 49,7003726 | M | A09_2010 | 2010 | **A09** |
| Daraina | 1 | **Bekaraoka** | BEK | -13,1727642 | 49,709958 | M | A10_2010 | 2010 | **A10** |
| Daraina | 1 | **Bekaraoka** | BEK | -13,1696851 | 49,6998082 | M | A11_2010 | 2010 | **A11** |
| Daraina | 1 | **Bekaraoka** | BEK | -13,1635381 | 49,7045234 | F | A13_2010 | 2010 | **A13** |
| Daraina | 1 | **Bekaraoka** | BEK | -13,1635407 | 49,7034574 | M | A14_2010 | 2010 | **A14** |
| Daraina | 1 | **Bekaraoka** | BEK | -13,1635457 | 49,7049016 | M | A15_2010 | 2010 | **A15** |
| Daraina | 1 | **Bekaraoka** | BEK | -13,1728688 | 49,7080599 | F | A17_2010 | 2010 | **A17** |
| Daraina | 1 | **Bekaraoka** | BEK | -13,15977 | 49,70356 | F | A19_2010 | 2010 | **A19** |
| Daraina | 1 | **Bekaraoka** | BEK | -13,1635407 | 49,7034574 | M | A20_2010 | 2010 | **A20** |
| Daraina | 1 | **Bekaraoka** | BEK | -13,1635343 | 49,7039821 | F | A21_2010 | 2010 | **A21** |
| Daraina | 1 | **Bekaraoka** | BEK | -13,1635526 | 49,7032645 | M | A22_2010 | 2010 | **A22** |
| Daraina | 1 | **Bekaraoka** | BEK | -13,1635547 | 49,7097836 | F | A23_2010 | 2010 | **A23** |
| Daraina | 1 | **Bekaraoka** | BEK | -13,1667237 | 49,7023731 | M | A25_2010 | 2010 | **A25** |
| Daraina | 1 | **Bekaraoka** | BEK | -13,1669008 | 49,7032412 | M | A26_2010 | 2010 | **A26** |
| Daraina | 1 | **Bekaraoka** | BEK | -13,1616512 | 49,7057354 | M | A28_2010 | 2010 | **A28** |
| Daraina | 1 | **Bekaraoka** | BEK | -13,185453 | 49,7353411 | M | K09_2011 | 2011 | **K09** |
| Daraina | 1 | **Bekaraoka** | BEK | -13,1837277 | 49,7291294 | F | K10_2011 | 2011 | **K10** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0904707 | 49,5780733 | F | A31_2010 | 2010 | **A31** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0864267 | 49,5766511 | F | A41_2010 | 2010 | **A41** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0935987 | 49,5852463 | M | A48_2010 | 2010 | **A48** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0885412 | 49,5821374 | F | A54_2010 | 2010 | **A54** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,088527 | 49,5794077 | M | A56_2010 | 2010 | **A56** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0885483 | 49,5779306 | F | A57_2010 | 2010 | **A57** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0904286 | 49,5811647 | F | A61_2010 | 2010 | **A61** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0945818 | 49,5827611 | M | A75_2010 | 2010 | **A75** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0921161 | 49,5829521 | M | A76_2010 | 2010 | **A76** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0811278 | 49,5780628 | M | B05_2010 | 2010 | **B05** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0791261 | 49,5786474 | M | B18_2010 | 2010 | **B18** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0791142 | 49,577714 | F | B20_2010 | 2010 | **B20** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0770213 | 49,5798725 | F | B30_2010 | 2010 | **B30** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0770237 | 49,5778653 | M | B35_2010 | 2010 | **B35** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0770731 | 49,5764099 | M | B38_2010 | 2010 | **B38** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0885482 | 49,5786636 | F | B49_2010 | 2010 | **B49** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0885322 | 49,5768253 | F | B50_2010 | 2010 | **B50** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0904855 | 49,5777759 | F | B58_2010 | 2010 | **B58** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0791212 | 49,5795312 | F | B62_2010 | 2010 | **B62** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0864786 | 49,5820878 | F | B70_2010 | 2010 | **B70** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0811425 | 49,5793298 | M | B78_2010 | 2010 | **B78** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0811159 | 49,5783042 | F | B80_2010 | 2010 | **B80** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0810386 | 49,5745138 | F | C05_2010 | 2010 | **C05** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0770447 | 49,5821807 | F | C09_2010 | 2010 | **C09** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0770109 | 49,5793158 | M | C10_2010 | 2010 | **C10** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0904855 | 49,5777759 | F | C16_2010 | 2010 | **C16** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0791227 | 49,5800046 | F | H14_2011 | 2011 | **H14** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,179051 | 49,733788 | F | K18_2011 | 2011 | **K18** |
| Daraina | 2 | **Solaniampilana** | SOL | -13,0791383 | 49,5823065 | F | K20_2011 | 2011 | **K20** |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Daraina | 3 | **Binara** | BIN | -13,2562391 | 49,6146327 | F | C24_2010 | 2010 | **C24** |
| Daraina | 3 | **Binara** | BIN | -13,2541764 | 49,619209 | M | C25_2010 | 2010 | **C25** |
| Daraina | 3 | **Binara** | BIN | -13,2409987 | 49,6315928 | F | C26_2010 | 2010 | **C26** |
| Daraina | 3 | **Binara** | BIN | -13,2406297 | 49,6316001 | F | C27_2010 | 2010 | **C27** |
| Daraina | 3 | **Binara** | BIN | -13,2394128 | 49,6322926 | F | C28_2010 | 2010 | **C28** |
| Daraina | 3 | **Binara** | BIN | -13,2486366 | 49,6136304 | F | C29_2010 | 2010 | **C29** |
| Daraina | 3 | **Binara** | BIN | -13,2485905 | 49,6138926 | F | C30_2010 | 2010 | **C30** |
| Daraina | 3 | **Binara** | BIN | -13,2491235 | 49,6164414 | F | C31_2010 | 2010 | **C31** |
| Daraina | 3 | **Binara** | BIN | -13,2491651 | 49,6166611 | F | C32_2010 | 2010 | **C32** |
| Daraina | 3 | **Binara** | BIN | -13,2489672 | 49,6093971 | F | C33_2010 | 2010 | **C33** |
| Daraina | 3 | **Binara** | BIN | -13,2491954 | 49,6200995 | F | C34_2010 | 2010 | **C34** |
| Daraina | 3 | **Binara** | BIN | -13,2411731 | 49,6308148 | M | C35_2010 | 2010 | **C35** |
| Daraina | 3 | **Binara** | BIN | -13,2400677 | 49,6308164 | F | C36_2010 | 2010 | **C36** |
| Daraina | 3 | **Binara** | BIN | -13,2394039 | 49,6311758 | M | C37_2010 | 2010 | **C37** |
| Daraina | 3 | **Binara** | BIN | -13,2388806 | 49,6313388 | F | C38_2010 | 2010 | **C38** |
| Daraina | 3 | **Binara** | BIN | -13,2376217 | 49,6323851 | M | C39_2010 | 2010 | **C39** |
| Daraina | 3 | **Binara** | BIN | -13,2371379 | 49,6327773 | F | C40_2010 | 2010 | **C40** |
| Daraina | 3 | **Binara** | BIN | -13,242489 | 49,6308486 | F | C41_2010 | 2010 | **C41** |
| Daraina | 3 | **Binara** | BIN | -13,275477 | 49,6183375 | F | C42_2010 | 2010 | **C42** |
| Daraina | 3 | **Binara** | BIN | -13,2740836 | 49,6192682 | F | C43_2010 | 2010 | **C43** |
| Daraina | 3 | **Binara** | BIN | -13,2490179 | 49,6067988 | F | C44_2010 | 2010 | **C44** |
| Daraina | 3 | **Binara** | BIN | -13,2489404 | 49,6069533 | F | C45_2010 | 2010 | **C45** |
| Daraina | 3 | **Binara** | BIN | -13,2573636 | 49,61258 | F | C47_2010 | 2010 | **C47** |
| Daraina | 3 | **Binara** | BIN | -13,2587102 | 49,6104777 | F | C48_2010 | 2010 | **C48** |
| Daraina | 4 | **Antsakay** | ANTSK | -13,0981708 | 49,7012452 | F | E29_2011 | 2011 | **E29** |
| Daraina | 4 | **Antsakay** | ANTSK | -13,0971042 | 49,7009616 | M | E30_2011 | 2011 | **E30** |
| Daraina | 4 | **Antsakay** | ANTSK | -13,1066879 | 49,6961521 | M | E32_2011 | 2011 | **E32** |
| Daraina | 4 | **Antsakay** | ANTSK | -13,1064491 | 49,698115 | M | E33_2011 | 2011 | **E33** |
| Daraina | 4 | **Antsakay** | ANTSK | -13,10604 | 49,7057154 | F | E34_2011 | 2011 | **E34** |
| Daraina | 4 | **Antsakay** | ANTSK | -13,1079375 | 49,7064623 | M | E35_2011 | 2011 | **E35** |
| Daraina | 4 | **Antsakay** | ANTSK | -13,0976872 | 49,7054341 | M | E37_2011 | 2011 | **E37** |
| Daraina | 4 | **Antsakay** | ANTSK | -13,0980403 | 49,7055257 | F | E38_2011 | 2011 | **E38** |
| Daraina | 4 | **Antsakay** | ANTSK | -13,0880468 | 49,7059062 | F | E39_2011 | 2011 | **E39** |
| Daraina | 4 | **Antsakay** | ANTSK | -13,0823489 | 49,7037737 | F | E43_2011 | 2011 | **E43** |
| Daraina | 4 | **Antsakay** | ANTSK | -13,0933862 | 49,7060271 | F | E51_2011 | 2011 | **E51** |
| Daraina | 4 | **Antsakay** | ANTSK | -13,097859 | 49,705452 | M | E53_2011 | 2011 | **E53** |
| Daraina | 4 | **Antsakay** | ANTSK | -13,0895231 | 49,706258 | M | E54_2011 | 2011 | **E54** |
| Daraina | 4 | **Antsakay** | ANTSK | -13,0844465 | 49,7044617 | F | E57_2011 | 2011 | **E57** |
| Daraina | 4 | **Antsakay** | ANTSK | -13,0818443 | 49,7034828 | F | E58_2011 | 2011 | **E58** |
| Daraina | 4 | **Antsakay** | ANTSK | -13,1066532 | 49,6959451 | M | E60_2011 | 2011 | **E60** |
| Daraina | 4 | **Antsakay** | ANTSK | -13,0933862 | 49,7060271 | F | E70_2011 | 2011 | **E70** |
| Daraina | 4 | **Antsakay** | ANTSK | -13,0919786 | 49,7066824 | M | E80_2011 | 2011 | **E80** |
| Daraina | 4 | **Antsakay** | ANTSK | -13,0858937 | 49,7051398 | M | F01_2011 | 2011 | **F01** |
| Daraina | 5 | **Ambohitsitondroina** | AMBO | -13,130372 | 49,4375665 | F | F19_2011 | 2011 | **F19** |
| Daraina | 5 | **Ambohitsitondroina** | AMBO | -13,1240654 | 49,4352483 | F | F20_2011 | 2011 | **F20** |
| Daraina | 5 | **Ambohitsitondroina** | AMBO | -13,1248819 | 49,4382609 | F | F30_2011 | 2011 | **F30** |
| Daraina | 5 | **Ambohitsitondroina** | AMBO | -13,1246875 | 49,4408135 | F | F37_2011 | 2011 | **F37** |
| Daraina | 5 | **Ambohitsitondroina** | AMBO | -13,1265024 | 49,4314002 | F | F50_2011 | 2011 | **F50** |
| Daraina | 5 | **Ambohitsitondroina** | AMBO | -13,1272824 | 49,4751074 | F | F65_2011 | 2011 | **F65** |
| Daraina | 5 | **Ambohitsitondroina** | AMBO | -13,1214704 | 49,4703544 | F | F66_2011 | 2011 | **F66** |
| Daraina | 5 | **Ambohitsitondroina** | AMBO | -13,1267614 | 49,4691025 | F | F70_2011 | 2011 | **F70** |
| Daraina | 5 | **Ambohitsitondroina** | AMBO | -13,1308558 | 49,4762748 | F | G04_2011 | 2011 | **G04** |
| Daraina | 5 | **Ambohitsitondroina** | AMBO | -13,1266418 | 49,4689476 | F | G13_2011 | 2011 | **G13** |
| Daraina | 6 | **Benanofy** | BEN | -13,0206996 | 49,5689716 | F | G27_2011 | 2011 | **G27** |
| Daraina | 6 | **Benanofy** | BEN | -13,020235 | 49,5685449 | F | G28_2011 | 2011 | **G28** |
| Daraina | 6 | **Benanofy** | BEN | -13,0030738 | 49,5852289 | M | G51_2011 | 2011 | **G51** |
| Daraina | 6 | **Benanofy** | BEN | -13,0158088 | 49,572918 | F | G52_2011 | 2011 | **G52** |
| Daraina | 6 | **Benanofy** | BEN | -13,0184091 | 49,5675445 | F | G61_2011 | 2011 | **G61** |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Daraina | 6 | **Benanofy** | BEN | -13,0179774 | 49,5673314 | M | G62_2011 | 2011 | **G62** |
| Daraina | 6 | **Benanofy** | BEN | -13,0145313 | 49,5649379 | F | G63_2011 | 2011 | **G63** |
| Daraina | 6 | **Benanofy** | BEN | -13,0133539 | 49,5722121 | F | G64_2011 | 2011 | **G64** |

## Annex B

**Table16-** Comparison of genetic diversity parameters obtained in the present study and in previous studies by other authors for all loci.

| **Author** | **Hapke *et al*** | | | | | | | | | | | | **Radespiel *et al*** | | | ***Wimmer et al*** | | | **Present study** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Species** | *M. murinus* | | | *M. berthae* | | | *M. griseorufus* | | | *M. rufus* | | | *M. murinus* | | | *M. murinus* | | | *M. tavaratra* | | |
| Locus | **He** | **Ho** | **NA** | **He** | **Ho** | **NA** | **He** | **Ho** | **NA** | **He** | **Ho** | **NA** | **He** | **Ho** | **NA** | **He** | **Ho** | **NA** | **He** | **Ho** | **NA** |
| Mm21 | 0.683 | 0.703 | 11 | 0.761 | 0.833 | 7 | 0.625 | 0.865 | 11 | 0.955 | 0.851 | 11 | - | - | - | - | - | - | 0.738 | 0.706 | 13 |
| Mm22 | 0.557 | 0.554 | 7 | 0.807 | 0.762 | 6 | 0.750 | 0.857 | 11 | 0.682 | 0.804 | 9 | - | - | - | - | - | - | 0.849 | 0.621 | 14 |
| Mm26b | 0.824 | 0.850 | 10 | na | na | na | 0.467 | 0.766 | 6 | 0.136 | 0.130 | 2 | - | - | - | - | - | - | 0.138 | 0.143 | 6 |
| Mm30 | 0.711 | 0.703 | 7 | 0.663 | 0.778 | 7 | 0.333 | 0.297 | 3 | 0.526 | 0.422 | 4 | - | - | - | - | - | - | 0.642 | 0.429 | 6 |
| Mm39 | 0.725 | 0.737 | 14 | 0.820 | 0.762 | 9 | 0.813 | 0.845 | 11 | 1.000 | 0.902 | 14 | - | - | - | - | - | - | 0.836 | 0.897 | 13 |
| Mm40 | 0.714 | 0.696 | 6 | 0.784 | 0.800 | 6 | 0.688 | 0.766 | 8 | 0.727 | 0.838 | 10 | - | - | - | - | - | - | 0.759 | 0.759 | 12 |
| Mm42 | 0.880 | 0.915 | 28 | 0.873 | 0.286 | 9 | 0.750 | 0.913 | 12 | na | na | na | - | - | - | - | - | - | 0.837 | 0.759 | 19 |
| Mm43b | 0.675 | 0.823 | 10 | 0.799 | 0.526 | 6 | 0.867 | 0.857 | 9 | 0.455 | 0.701 | 5 | - | - | - | - | - | - | 0.578 | 0.517 | 13 |
| Mm51 | 0.165 | 0.154 | 3 | 0.557 | 0.524 | 3 | 0.688 | 0.589 | 3 | 0.591 | 0.685 | 7 | - | - | - | - | - | - | 0.327 | 0.345 | 3 |
| Mm58 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.883 | 0.862 | 13 |
| Mm60 | 0.757 | 0.812 | 9 | mm | mm | mm | 0.875 | 0.929 | 13 | na | na | na | - | - | - | - | - | - | mm | mm | mm |
| C1P3 | - | - | - | - | - | - | - | - | - | - | - | - | 0.870 | 0.610 | 21 | - | - | - | 0.905 | 0.929 | 24 |
| Mm02 | - | - | - | - | - | - | - | - | - | - | - | - | 0.620 | 0.620 | 10 | - | - | - | 0.687 | 0.552 | 9 |
| Mm03 | - | - | - | - | - | - | - | - | - | - | - | - | 0.840 | 0.850 | 15 | - | - | - | 0.823 | 0.862 | 8 |
| Mm07 | - | - | - | - | - | - | - | - | - | - | - | - | 0.230 | 0.220 | 5 | - | - | - | 0.803 | 0.821 | 6 |
| Mm08 | - | - | - | - | - | - | - | - | - | - | - | - | 0.920 | 0.930 | 29 | - | - | - | 0.884 | 0.931 | 17 |
| Mm10 | - | - | - | - | - | - | - | - | - | - | - | - | 0.920 | 0.910 | 22 | - | - | - | na | na | na |
| MmF3 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.908 | 0.866 | 18 | 0.626 | 0.593 | 5 |
| Mm06 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.896 | 0.902 | 16 | mm | mm | mm |
| MmF6 | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | 0.91 | 0.948 | 16 | mm | mm | mm |

He-expected heterozygosity; Ho-observed heterozygosity; NA-number of alleles
mm-monomorphic; na-no amplification; - no data

(Hapke et al., 2003; Radespiel et al., 2001; Wimmer et al, 2002)

Only one site (SOL) was chosen for this table in order to facilitate comparison as the values from the other authors are also representative of only one sampling location.

## Annex C

Qiagen BTK DNA extraction

1. Place the tissue in a 1.5ml tube
2. Add **300 µl buffer ATL**, **20 µl proteinase K** and **20 µl 1M DTT**

   *Note 1: Adapt the quantity of these products according to the tissue size:*

| | Prot K | DTT | AE |
|---|---|---|---|
| **Small** | 20 | 20 | 55 |
| **Medium** | 30 | 20 | 70 |
| **Large** | 30 | 20 | 85 |

   *Note 2: Follow always the next order:* ***Tissue + Prot K + buffer ATL + DTT***

3. Vortex **10s**
4. Incubate at **56ºC** with shaking at **900rpm overnight**
5. Centrifuge briefly
6. Add **300 µl buffer AL**

   *A white precipitate may form when buffer AL is added to buffer ATL. The precipitate does not interfere with the procedure and will dissolve during incubation*
7. Vortex for **10s**
8. Incubate at **70ºC** with shaking at **900rpm for 10 min**
9. Add **150µl ethanol (96-100%)**
10. Incubate **5m** at room temperature
11. Carefully transfer the supernatant (400 µl) to the QIAamp column (in a 2 ml collection tube)
12. Centrifuge at **8000 rpm for 1min**
13. Carefully transfer the last 400 µl of supernatant to the QIAamp column
14. Centrifuge at **8000 rpm for 1min**
15. Place the column in a clean 2 ml collection tube and discard the collection tube containing the flow-through
16. Add **500 µl buffer AW1** and centrifuge at **8000 rpm for 1min**
17. Empty the collection tube, clean the top on a paper tissue and put column back in place
18. Add **500 µl buffer AW2** and centrifuge at **8000 rpm for 1min**
19. Transfer the column to a new collection tube.
20. Add **700 µl of ethanol (96-100%)** and centrifuge at **8000 rpm for 1min**.
21. Empty the flow-through and clean the top of the collection tube on a paper tissue
22. Centrifuge at full speed **14000 rpm for 3min** to dry the membrane completely
23. Place the column in a clean 1.5 ml tube, open the lid of the column and **incubate at room temperature (15ºC-25ºC) for 10 min or at 56ºC for 3 min**
24. Apply **40-50µl AE buffer** or distilled buffer water, to the center of the membrane
25. Incubate at room temperature for **10 min**.
26. Centrifuge at full speed **14000 rpm for 2 min**
27. Repeat steps 24 and 25 using a new 1.5mL tube
28. Measure the DNA concentration with the **Nanodrop** and choose the best sample to use frequently (stock sample) and keep the other one as a backup sample in a -80ºC freezer

    *Note: You can then prepare a plate of 96 wells with DNA at a concentration of 20-30ng/uL to be used in DNA amplifications.*